Adaptationen von T Helferlymphozyten an chronische Entzündungen

Habilitationsschrift zur Erlangung der Lehrbefähigung
für das Fach: Biologie

vorgelegt dem Rat der Lebenswissenschaftlichen Fakultät der Humboldt-Universität zu Berlin
von

Dr. rer. nat. Hyun-Dong Chang

Berlin, den 02. März 2017

Prof. Dr.-Ing. Dr. Sabine Kunst
Präsidentin
der Humboldt-Universität zu Berlin

Prof. Dr. Bernhard Grimm
Dekan der
Lebenswissenschaftlichen Fakultät

Gutachterinnen und Gutachter:
1. Prof. Dr. Michael Lohoff, Philipps Universität Marburg
2. Prof. Dr. Hans-Martin Jäck, Friedrich-Alexander Universität Erlangen-Nürnberg
3. Prof. Dr. Hans-Dieter Volk, Charité – Universitätsmedizin Berlin

verteidigt am 12. Januar 2018

Inhaltsverzeichnis

1 Einleitung

Das adaptive Immunsystem ist in der einzigartigen Lage mit einer spezifischen Abwehrreaktion Krankheitserreger zu bekämpfen. Die zelluläre Basis dieser Immunreaktion sind die B und T Lymphozyten, die jeweils Antigenrezeptoren einer einzigen Spezifität tragen. Während der Entwicklung der B und T Lymphozyten wird durch die zufällige Rekombination von Gensegmenten, der VDJ-Rekombination, eine hohe Diversität an Antigenrezeptoren generiert. Im Laufe einer Infektion werden die Lymphozyten aktiviert, die mit ihrem spezifischen Rezeptor den Krankheitserreger bzw. Bestandteile oder Produkte des Krankheitserreger erkennen. In der sich ergebenden Immunreaktion differenzieren die aktivierten Lymphozyten und passen sich so an den Krankheitserreger an, um ihn effektiver bekämpfen zu können. Während das Antigen selbst die Selektion des Antigenrezeptors, d.h. des B und T Zellrezeptors, bestimmt, spielen die T Helfer (Th)-Lymphozyten eine ganz entscheidende Rolle die die Immunreaktion insgesamt zu fördern und zu steuern. Th Lymphozyten induzieren über CD40L-CD40 Interaktion bei aktivierten B Lymphozyten die somatische Hypermutation, die letztendlich zu einer Vervielfachung der Affinität des B Zellrezeptors (BCR) gegenüber dem Antigen führt, und steuern über Zytokine den Immunglobulinklassenwechsel bei B Zellen, der die Effektorfunktion der Antikörper bestimmt. Darüber hinaus regeln Th Zellen über die Expression von Zytokinen und Chemokinen die Aktivierung von zytotoxischen T Zellen, sowie die Rekrutierung und Aktivierung von Makrophagen, Monozyten und Granulozyten. Dies bedeutet, dass Th Zellen durch ihre zentrale Funktion den Verlauf der Immunantwort kontrollieren. In der vorliegenden Arbeit wurde untersucht, inwiefern Th Zellen selbst während ihrer Aktivierung instruiert werden und wie sie sich selbst an die Entzündung anpassen. Insbesondere im Kontext einer chronischen Entzündung scheinen diese Anpassungen, oder Adaptationen, dazu zu führen, dass Th Zellen zur Aufrechterhaltung der Entzündung und zur Pathogenese beitragen.

1.1 T-Helferzelldifferenzierung

1.1.1 T-Helferzelltypen

Th Zellen zeichnen sich nach ihrer Aktivierung in erster Linie durch die Expression von verschiedenen Zytokinen aus. Zytokine dienen als Botenstoffe, um andere Zellen des Immunsystems zu aktivieren und zu instruieren. Es wurde ursprünglich gezeigt, dass Th Zellen anhand ihrer Zytokinexpression in zwei Subtypen unterteilt werden können: Th Typ 1 (Th1)

Zellen, die Interferon-γ (IFNγ) und Tumornekrosefaktor (TNF) exprimieren, und Th2 Zellen, die Interleukin-4 (IL-4), IL-5 und IL-13 exprimieren [1]. Die unterschiedlichen Th-Zelltypen beeinflussen das Wesen der Immunantwort. Generell wird postuliert, dass eine durch Th1-Zellen geförderte Immunantwort besonders effektiv gegen intrazelluläre Erreger ist, indem sie durch die Ausschüttung von IFNγ Makrophagen und zytotoxische T Zellen aktivieren. Im Gegensatz dazu wird Th2-Zellen eher eine Rolle bei der Immunantwort gegen multizelluläre Parasiten zugeschrieben. Durch die Expression von IL-4 fördern sie den Immunglobulinklassenwechsel der B Zellen zu IgE [2] und aktivieren durch IL-5 eosinophile Granulozyten [3], beides wichtige Mechanismen, die eine effektive Verteidigung gegen Parasiten ausmachen. Mittlerweile wurden weitere Th-Zellsubtypen identifiziert. Die prominentesten Vertreter sind wahrscheinlich die sogenannten Th17-Zellen, die sich durch die Expression von IL-17A, IL-17F, IL-21 und IL-22 auszeichnen [4]. Th17-Zellen sind insbesondere für die Immunantwort gegen extrazelluläre Bakterien, wie z.B. *Citrobacter rodentium* [5], und *Pilzen*, wie *Candida albican*s [6], von wichtiger Bedeutung. Andere Th Zellsubtypen, wie z.B. den Th9-Zellen [7, 8], Th22-Zellen [9] oder Tr1-Zellen [10] wurden im Kontext von Atemwegsentzündung, Darmhomöostase und chronischer Infektion beschrieben.

1.1.2 Instruierende Signale der T-Helferzelldifferenzierung

Zu welchem Subtyp eine Th-Zelle differenziert bzw. welche Zytokine eine Th-Zelle nach ihrer Aktivierung exprimiert hängt von instruierenden Signalen ab, die die Th-Zelle von der antigen-präsentierenden Zelle (APC) und anderen benachbarten Zellen bekommt. Während für die Th1-Differenzierung gezeigt wurde, dass IL-12 ein instruierendes Signal ist [11-13], spielt IL-4 eine zentrale Rolle für die Th2-Differenzierung [14-16]. TGFβ (tumor growth factor β) [17, 18], IL-6 [19] und IL-1β [20] wurden als Signale identifiziert, die die Differenzierung von Th17-Zellen fördern. Diese instruierenden Signale müssen zeitlich mit der T-Zellrezeptoraktivierung zusammenfallen, um die Differenzierung der aktivierten Th-Zelle einzuleiten [21].

Binden die Zytokine an ihre Rezeptoren führt das zu einer Dimerisierung der Rezeptorketten und der Aktivierung der rezeptor-assoziierten Janus-Kinasen (JAKs). Die JAKs phosphorylieren daraufhin Tyrosine in der Rezeptorkette, die dadurch die *signal transducers and activators of transcription* (STATs) binden können, die wiederum selbst von den JAKs phosphoryliert werden und dimerisieren. Im dimerisierten Zustand translozieren die STATs in den Zellkern und transaktivieren die Transkription von Genen, die für die jeweiligen Zytokine kodieren [22]. Zusätzlich zu den Zytokingenen wird auch die Transkription von Schlüsseltranskriptionsfaktoren durch die STATs angeschaltet. IL-12 führt nach Bindung an den IL-12-Rezeptor zur Aktivierung von STAT4, das neben der Transkription von *Ifng* auch die Expression des T-bet-kodierenden

Gens *Tbx21* initiiert [13, 22]. Die Bindung von IL-4 an seinen Rezeptor führt zur Aktivierung von STAT6, das die Transkription von *Il4*, sowie dem Th2 Transkriptionsfaktor *Gata3* initiiert [23]. Analog dazu führt IL-6-vermittelte STAT3 Aktivierung zur Expression des Transkriptionsfaktors RORγt, was die Th17 Differenzierung induziert [24].

T-bet, GATA-3 und RORγt gelten als sogenannte Mastertranskriptionsfaktoren, da ihre Funktion notwendig und ausreichend ist, um die Differenzierung von Th Zellen in Th1, Th2, oder Th17 Zellen zu induzieren [25-27].

1.2 Das Zytokingedächtnis

Bei ihrer Primäraktivierung durchlaufen Th-Zellen ein bestimmtes Zytokinexpressionsmuster, welches die jeweilige Phase der T-Zellaktivierung bestimmt. Sofort nach Aktivierung exprimiert die Th-Zelle IL-2, welches auf autokrine Weise die Proliferation und Expansion von aktivierten Th Zellen fördert [28]. In den darauffolgenden Tagen exprimieren die Th-Zellen dann abhängig von den instruierenden Signalen ihre Effektorzytokine, wie z.B. IFNγ, und fördern anschließend durch die Expression von IL-10 die Beendigung der Immunreaktion [29].

Werden Th-Zellen, die schon eine Aktivierung durchgemacht haben, erneut aktiviert, exprimieren sie in der Regel die gleichen Zytokine, die sie während ihrer Primäraktivierung exprimiert haben [30]. Im Gegensatz zur Primäraktivierung tun sie dies innerhalb von Stunden [31]. Bei der Reaktivierung reicht die Stimulation über den T-Zellrezeptor aus, um die Expression der Zytokine auszulösen, ohne dass erneut instruierende Signale benötigt werden. Sie haben ein sogenanntes Zytokingedächtnis ausgebildet. Dieses Zytokingedächtnis beruht auf der stabilen epigenetischen Prägung der entsprechenden Zytokingene und der stabilen Expression von bestimmten Transkriptionsfaktoren. Erste Hinweise für die epigenetische Prägung kamen von Studien, die zeigten, dass Th-Zellen für die Etablierung eines Zytokingedächtnisses mindestens die S-Phase der ersten Zellteilung nach Aktivierung durchlaufen haben muss [21]. Während der Replikation der DNA in der S-Phase ist die DNA zugänglich für epigenetische Modifikationen, wie z.B. der Zytosin-Methylierung [32]. Weiterhin interagiert der chromatin-remodellierende Faktor *Brahma-related gene 1* (Brg1) präferentiell während der S-Phase mit der DNA [33]. Brg1 ist eine Komponente des SWI/SNF-Chromatinremodelierkomplexes und spielt eine wichtige Rolle bei der epigenetischen Prägung von Zytokingenen durch Histonmodifikationen [34, 35]. In naiven Th-Zellen liegen die meisten Zytokingene in einer „geschlossenen“ Konfiguration vor, d.h. Zytosine regulatorischer Elemente, wie z.B. Promotoren und Enhancer, sind hypermethyliert und die Histone tragen repressorische Modifikationen. In der Regel liegen die Zytokingene in solchen Fällen in heterochromatischer Form vor, unzugänglich

für Transkriptionsfaktoren [36-39]. Nach Aktivierung der T-Zelle in Abhängigkeit von instruierenden Signalen können sogenannte Pionierfaktoren an andernfalls geschlossenes Chromatin binden, chromatin-remodellierende Faktoren und weitere Transkriptionsfaktoren rekrutieren und die schnelle und transiente Expression bestimmter Zytokingene ermöglichen. Vieles deutet daraufhin, dass die STATs diese Funktion übernehmen [22, 40, 41]. STATs induzieren gleichzeitig die Expression der Mastertranskriptionsfaktoren, die ihrerseits an geschlossenes Chromatin binden können, weitere chromatin-remodellierende Faktoren rekrutieren und die Zytokingene dauerhaft durch DNA-Demethylierung und Histonmodifizierung prägen [42, 43]. Im Folgenden wird kurz auf spezifische Mechanismen bei der Prägung verschiedener Zytokingene eingegangen.

1.2.1 Induktion des IL-4-Gedächtnisses

Für IL-4 konnten wir zeigen, dass GATA-3 an eine phylogenetisch konservierte Bindestelle im ersten Intron des *Il4*-Gens bindet und ausgehend von dort wahrscheinlich die epigenetische Prägung des *Il4*-Gens in Form von CpG-Demethylierung einleitet [38]. Während der fortschreitenden Th2-Differenzierung breitet sich die CpG-Demethylierung über das ganze *Il4*-Gen aus, wobei bestimmte phylogenetisch konservierte regulatorische Elemente, wie z.B. die CNS1 und CNS2, koordiniert zur stabilen Prägung der IL-4-Expression beitragen [30, 39]. Zusätzlich zur epigenetischen Prägung des *Il4*-Gens kommt es zu einer transkriptionellen Prägung, bei der die Expression von GATA-3 stabilisiert wird. GATA-3 bindet an seinen eigenen Promoter und induziert seine eigene Expression [44, 45]. Dadurch wird die GATA-3-Expression verstärkt und resultiert sowohl in einer stabilen GATA-3-Expression [46] als auch einem stabilisierten Th2-Phänotyps. Während etablierte Th2 Zellen auch nach Deletion des *Gata3*-Gens noch IL-4 exprimieren, scheint die Expression der Th2-Zytokine IL-5 und IL-13 komplett von der kontinuierlichen Gegenwart von GATA-3 abhängig zu sein [47]. Anscheinend wird die epigenetische Prägung des *Il4*-Gens auch ohne GATA-3 aufrecht erhalten.

1.2.2 Induktion des IFN-γ-Gedächtnisses

Ein ähnlicher Mechanismus, wie der, der zur Th2-Differenzierung führt, konnte für die Th1-Differenzierung und der Prägung des *Ifng*-Gens gezeigt werden. Bei der Th1-Differenzierung induziert die Bindung von IFN-γ an den IFN-γ-Rezeptor die Aktivierung von STAT1. STAT1 verursacht die transiente Expression von T-bet, was die Expression von *Ifng* initiiert [48]. Ein weiteres wichtiges instruktives Signal für die Th1-Differenzierung ist IL-12 [49]. Jedoch wird die Expression der induzierbaren Signalkette des IL-12-Rezeptors, der β2-Kette des IL-12-Rezeptors (IL12Rβ2) transkriptionell durch T-Zellrezeptoraktivierung inhibiert wird [13]. Erst

nach Verminderung des T-Zellrezeptorsignals zu einem späteren Zeitpunkt der Immunreaktion kann IL12Rβ2 exprimiert und die T-Zelle auf IL-12 reagieren. Die Bindung von IL-12 an seinen Rezeptor führt zur Aktivierung von STAT4, welches auch die Expression von T-bet induziert. Dieser späte Zeitpunkt der T-bet-Expression fällt zeitlich mit der Expression der Faktoren *H2.0-like Homeobox Protein* (Hlx) und *Runt-related transcription factor 3* (Runx3) zusammen [13]. Hlx und Runx3 sind Transkriptionsfaktoren, die zusammen mit T-bet das *Ifng*-Gen dauerhaft für die Reexpression in nachfolgenden T-Zellaktivierungen [50, 51].

1.2.3 Das IL-17-Gedächtnis

Bei Th17-Zellen sorgen die instruktiven Signale TGF-β und IL-6 für die aktivierenden Histonmodifikationen, Histone H3-Hyperazetylierung und Lys-4 Trimethylierung im *Il17A*- und *Il17F*-Lokus [52]. Allerdings scheint die epigenetische Prägung von Th17-Zellen nicht sonderlich stabil zu sein und geht in Anwesenheit von widrigen Differenzierungssignalen, wie z.B. IL-12, verloren [53]. Dennoch gibt es Mechanismen, die den Th17-Phänotyp stabilisieren: IL-21 wird von Th17-Zellen exprimiert, die wiederum selbst den IL-21-Rezeptor auf ihrer Oberfläche tragen. IL-21 führt so zu einer autokrinen Aktivierung von STAT3 und einer positiven Rückkopplungsschleife zur Festigung der RORγt- und IL-17-Expression [54]. Weiterhin wird durch die STAT3-Aktivierung in Th17-Zellen die Expression des IL-23-Rezeptors induziert. IL-23 selbst trägt zur weiteren Stabilisierung von Th17-Zellen bei [55, 56].

1.2.4 Das IL-10 Gedächtnis

Das Zytokin IL-10 kann keinem spezifischen Th-Zellsubtyp zugeordnet werden und gilt generell als immunsuppressives Zytokin, welches unter anderem die Antigen-Präsentation, effiziente Aktivierung von T-Zellen und Zytokinexpression inhibiert [57-61]. Allerdings fördert IL-10 auch das Überleben und die Differenzierung von B-Zellen zu Plasmazellen [62, 63] und kann so je nach Kontext auch insbesondere B-zell-vermittelte Immunantworten fördern. Die generelle Abwesenheit von IL-10 [64], aber auch die selektive Deletion von des *Il10*-Gens in T-Zellen [65], ist mit erhöhter Immunpathologie verbunden, weshalb IL-10 wahrscheinlich sowohl von Th1 als auch von Th2 und Th17-Zellen exprimiert wird [31, 66, 67]. IL-10 wird während der Primäraktivierung von Th-Zellen im Vergleich zu den entzündungsfördernden Zytokinen, wie IFN-γ, relativ spät exprimiert [29]. Aufgrund der immunsuppressiven Eigenschaften von IL-10 würde diese späte IL-10-Expression eine effektive T-zellvermittelte Immunantwort erlauben. Bei der Sekundärantwort verhält sich IL-10 in den verschiedenen Th-Zelltypen unterschiedlich. Das *Il10*-Gen wird in Th-Zellen relativ spät bzw. gar nicht funktionell geprägt. In Th2-Zellen werden mehrere Aktivierungszyklen in Gegenwart des instruierenden Signals IL-4 benötigt, bis IL-10

unabhängig von IL-4 exprimiert wird [31]. In Th2-Zellen wird *Il10* in Abhängigkeit von GATA-3, welches direkt an den *Il10*-Promoter bindet, epigenetisch geöffnet [66, 68]. Warum mehrere Aktivierungszyklen notwendig sind ist bisher noch unbekannt. In Th1-Zellen wird IL-10 durch IL-12 [66] und Notch induziert [69]. Th1-Zellen entwickeln kein Gedächtnis für IL-10-Expression und benötigen die kontinuierliche Gegenwart von IL-12 bzw. STAT4-induzierenden Signalen, um IL-10 zu re-exprimieren [66].

1.3 Th-Zellen bei chronischen Entzündungen

Wie bereits erwähnt, fördern Th1-, Th2- und Th17-Zellen aufgrund ihrer jeweiligen Effektorfunktionen Immunantworten, die gegen unterschiedliche Pathogene effektiv sind. Die Regulation, welche Th-Zelldifferenzierung bei einer Immunatwort induziert wird, geschieht in der Regel über Zellen des angeborenen Immunsystems, den antigen-präsentierenden Zellen. Über bestimmte Rezeptoren, den *pattern recognition receptors* (PRR), die konservierte Strukturen, wie z.B. unmethylierte DNA, Flagellen oder Lipopolysaccharide von Pathogenen erkennen, wird die Expression von instruierenden Zytokinen und kostimulierenden Molekülen auslöst. Dabei ist die Induktion der optimalen Th-Zelldifferenzierung für den Verlauf der Immunatwort wichtig. Kommt es aufgrund genetischer oder umweltbedingter Einflüsse zu einer fehlgeleiteten Th-Zellantwort, kann es katastrophale Folgen haben. Ein klassisches Beispiel hierfür ist das *Leishmania*-Infektionsmodell in der Maus [70] und die Infektion mit *Mycobacterium leprae* beim Menschen [71]. In beiden Fällen wird eine Th1-Antwort benötigt, um den intrazellulären Krankheitserreger effektiv zu bekämpfen. Ein wichtiger Effektormechanismus für die Bekämpfung dieser Erreger ist die Aktivierung infizierter Makrophagen durch IFN-γ, in denen dann die Fusion der bakterien-haltigen Vesikel mit Lysosomen ausgelöst wird und so der Erreger abgetötet wird. In einigen Fällen kann es jedoch zu einer chronischen Entzündung kommen bei der der Erreger nicht effektiv bekämpft wird. Dies kann z.B. eine Th2-dominierte Immunantwort sein, die nicht optimal in der Lage ist kritische Effektormechanismen, wie der Aktivierung von Maktrophagen, auszulösen. Dadurch kann der Erreger persistieren und es kommt zu einer chronischen Aktivierung des Immunsystems mit der damit verbundenen Immunpathologie.

Aus bisher noch nicht geklärten Gründen kann es auch zu Immunantworten gegen nicht-pathogen-assoziierte Antigene, wie z.B. Allergene oder Selbstantigene kommen. Normalerweise werden solche Antigene ignoriert oder die Immunantworten werden über zentrale und periphere Toleranzmechanismen, wie der Negativselektion autoreaktiver Th-Zellen im Thymus oder

Inhibition durch regulatorische T-Zellen, unterdrückt. Werden diese Toleranzmechanismen umgangen bzw. durchbrochen kann es je nach Differenzierung der beteiligten Th-Zellen zu einer pathologischen chronischen Entzündung kommen.

Allergien, insbesondere Hypersensitivitätsreaktionen vom Typ 1, sind Immunreaktionen gegen Allergene, die über spezifische Antikörper vom Isotyp IgE vermittelt werden. Der Immunglobulinklassenwechsel zu IgE wird durch IL-4 induziert. Es spielen daher Th2-Zellen eine zentrale Rolle bei einer allergischen Reaktion. Sowohl in Mäusen mit experimentellem Asthma [72] als auch in Menschen [73] kann durch die Inhibition der Th2-Funktion mittels Blockade von GATA-3 eine klinische Verbesserung der asthmatischen Entzündung herbeigeführt werden.

Kommt es zu einer pathologischen Immunreaktion gegen körpereigene Strukturen, oder Autoantigenen, spricht man von einer Autoimmunkrankheit. Die starke Assoziation von vielen Autoimmunkrankheiten mit bestimmten MHC Allelen deutet daraufhin, dass eine T-Zell-vermittelte adaptive Immunantwort eine wichtige Rolle spielt. Die Depletion von $CD4^+$ T-Zellen in kleinen, offenen klinischen Studien bei rheumatoider Arthritis [74-76] aber auch bei chronisch-entzündlichen Darmerkrankungen [77] hat gezeigt, dass Th-Zellen eine kritische Rolle bei der Aufrechterhaltung von chronischen Entzündungen spielt. Allerdings ließ sich dieser therapeutische Effekt nicht in größeren placebo-kontrollierten Studien reproduzieren [78, 79]. Heute geht man davon aus, dass durch die anti-$CD4^+$-Antikörper vermittelte Depletion der Th-Zellen auch die anti-inflammatorischen regulatorischen T-Zellen eliminiert wurden [80] und sich daher die klinische Wirksamkeit der T-Zelldepletion in Grenzen hielt. Eine weitere Nebenwirkung der anti-CD4-Behandlung war die langanhaltende Depletion der Th-Zellen, auch lange nach Absetzen der Therapie [75, 81], was eine weitere Anwendung dieses eher unselektiven Therapieansatzes ausschloss.

Allerdings zeigen Tiermodelle nach wie vor, dass Th-Zellen eine wichtige Rolle bei chronischen Entzündungen spielen. In vielen Tiermodellen für Autoimmunkrankheiten reicht oft allein der adoptive Transfer von autoreaktiven Th-Zellen oder die Aktivierung von autoreaktiven Th Zellen durch Immunisierung aus, um eine Entzündung in dem Gewebe, welches das Autoantigen exprimiert, auszulösen und aufrechtzuerhalten, wie z.B. im Darm [82], im Pankreas [83], im zentralen Nervensystem [84] oder in den Gelenken [85]. Dabei ist die Differenzierung der Th-Zellen auschlaggebend, ob es zu einer pathogenen Entzündung kommt.

Sowohl IL-12 als auch IL-23 wurden als wichtige entzündungsfördernde Zytokine in diversen Tiermodellen identifiziert. Die primäre Rolle, die diesen beiden Zytokinen bei der Induktion autoimmuner Entzündungen zugeschrieben wird, ist die Instruktion der Th1- bzw. Th17-Differenzierung. Die antikörper-vermittelte oder genetische Blockade von IL12p40, der

gemeinsamen Untereinheit von IL-12 und IL-23 [86], verhindert die Induktion einer Reihe von Autoimmunkrankheiten in Mäusen [87-89]. Interessanterweise, führt die Blockade bzw. Defizienz der IL-12 Untereinheit IL12p35 oder der IL-23 Untereinheit IL12p19 zu unterschiedlichen Empfänglichkeiten von Mäusen gegenüber der Induktion von Autoimmunentzündungen [90] und deutet daraufhin, dass IL-12 und IL-23 unterschiedliche pathogene Funktionen in unterschiedlichen Autoimmunmodellen haben.

In Tiermodellen für z.B. Typ 1 Diabetes, multipler Sklerose, und chronischer Darmentzündung konnte die pathogene Rolle von Th1- bzw. Th17-Zellen durch die zelltyp-spezifische Deletion oder Blockade von T-bet, RORγt, IFNγ oder IL-17 bestätigt werden [27, 91-93]. Auch im entzündeten Gewebe bei Patienten mit chronisch-entzündlichen Krankheiten werden gehäuft Th Zellen gefunden, die IFN-γ und IL-17 bzw. T-bet und RORγt exprimieren [94]. Viele Th Zellen *in vivo*, sowohl in Entzündungsmodellen der Maus als auch bei Patienten, weisen einen Mischphänotyp auf, d.h. sie exprimieren sowohl IFN-γ als auch IL-17 [95]. In Mausstudien wurde gezeigt, dass Th1- und Th17-Zellen unter gewissen Umständen kooperieren, um eine Entzündung auszulösen [96, 97]. Es könnte daher sein, dass gerade die Th-Zellen, die gewissermaßen, die entzündungsfördernden Eigenschaften von Th1- und Th17-Zellen in sich vereinen, besonders pathogen sind.

2 Fragestellung

Th-Zellen differenzieren je nach den Signalen, die sie während ihrer erstmaligen Aktivierung bekommen in unterschiedliche Th-Zelltypen mit spezialisierten Funktionen, die sie optimal für die Verteidigung gegen Pathogene ausstatten. Im Falle einer Autoreaktivität können gerade diese Funktionen zur Pathogenese von Autoimmunkrankheiten beitragen.

Diese Arbeit beschäftigt sich mit der Frage, wie insbesondere pro-inflammatorische Eigenschaften von Th-Zellen epigenetisch und funktionell geprägt werden. Desweiteren stellen wir uns die Frage, ob Th-Zellen im Kontext einer chronischen Entzündung Eigenschaften erlangen, die zum einen zur Perpetuation der Entzündung beitragen, und ob diese errungenen Eigenschaften nutzbar sind pathogene Th-Zellen selektiv therapeutisch anzugehen.

3 Eigene Arbeiten

Im Folgenden werden einige ausgewählten Arbeiten näher beschrieben.

3.1 In vivo differenzierte Th17-Zellen haben ein stabiles Gedächtnis für die IL-17-Reexpression

Lexberg MH, Taubner A, Förster A, Albrecht I, Richter A, Kamradt T, Radbruch A*, **Chang HD***. 2008. Th memory for interleukin-17 expression is stable *in vivo*. *Eur J Immunol.* 38(10):2654-64.

Th17-Zellen wurden insbesondere mit chronischen Entzündungen wie Multipler Sklerose, chronisch-entzündlicher Darmentzündung und Psoriasis in Verbindung gebracht. Sie zeichnen sich durch die Expression von IL-17 aus und wurden als weiterer Effektor-Th-Zelltyp neben Th1- und Th2-Zellen beschrieben.

Wir haben die Stabilität des Zytokin-Gedächtnisses für IL-17A in Th17-Zellen analysiert. Um die Stabilität der IL-17-Expression auf Einzelzellebene untersuchen zu können, haben wir in Zusammenarbeit mit Miltenyi Biotec den IL-17-Sekretionsassay entwickelt, der es erlaubt lebende Th-Zellen anhand der Sekretion von IL-17 zu analysieren. Hierfür kommt eine heterodimere Affinitätsmatrix zum Einsatz, die einerseits über CD45 an die Zelloberfläche gebunden wird und andererseits sezerniertes IL-17A bindet. So können die Zellen identifiziert und isoliert werden, die zu dem gegebenen Zeitpunkt IL-17 sezerniert haben. Wir konnten zeigen, dass *in vitro*, mit TGF-β, IL-6 und IL-23 differenzierte Th17-Zellen in nachfolgenden Restimulationen ihre IL-17A Expression verlieren, wenn diese instruierenden Signale fehlen. In Abwesenheit von instruierenden Signalen verloren die *in vitro* differenzierten Th17 Zellen auch die Expression der Transkriptionsfaktoren RORγt und RORα. In Gegenwart vom Th1-instruierenden Signal IL-12, bzw. vom Th2-instruierenden Signal IL-4 differenzierten die *in vitro* differenzierten Th17-Zellen zu *bona fide* Th1- bzw. Th2-Zellen. Im Gegensatz dazu behielten Th-Zellen, die direkt *ex vivo* aus immunisierten Mäusen anhand ihrer IL-17A Expression isoliert wurden, ihre IL-17A-Expression, auch unter Th1- oder Th2-induzierenden Bedingungen. *In vivo* differenzierte Th17-Zellen behielten ihre Expression von RORγt und RORα und hatten weder T-bet noch GATA-3 bzw. IFNγ oder IL-4 hochgeregelt.

Diese Ergebnisse haben gezeigt, dass Th-Zellen während ihrer Differenzierung zu Th17-Zellen *in vivo* im Vergleich zur *in vitro* Differenzierung anscheinend noch weiteren noch nicht identifizierten Signalen ausgesetzt sind, die zur Stabilität des Zytokin-Gedächtnisses für IL-17A

beitragen. Des Weiteren weisen die Ergebnisse darauf hin, dass man keine sicheren Rückschlüsse über das Verhalten von *in vivo* generierten Th17-Zellen durch Untersuchungen von *in vitro* differenzierten Th17-Zellen schließen darf.

Frontline

European Journal of Immunology

Th memory for interleukin-17 expression is stable *in vivo*

Maria H. Lexberg[1], Annegret Taubner[2], Anna Förster[3], Inka Albrecht[1], Anne Richter[3], Thomas Kamradt[2], Andreas Radbruch[1] and Hyun-Dong Chang*[1]*

[1] German Rheumatism Research Center Berlin, Berlin, Germany
[2] Institute of Immunology, Friedrich Schiller University Jena, Medical School, Germany
[3] Miltenyi Biotec GmbH, Bergisch Gladbach, Germany

Based on the memory for the re-expression of certain cytokine genes, different subsets of Th cells have been defined. In Th type 1 (Th1) and Th2 memory lymphocytes, the genes for the cytokines interferon-γ and interleukin (IL)-4 are imprinted for expression upon re-stimulation by the expression of the transcription factors T-bet and GATA-3, respectively, and epigenetic modification of the cytokine genes. In Th17 cells, IL-17 expression is dependent on the transcription factors RORγt and RORα. Here, we analyze the stability and plasticity of IL-17 memory in Th17 cells. We have developed a cytometric IL-17 secretion assay for the isolation of viable Th cells secreting IL-17. For Th17 cells generated *in vitro*, IL-17 expression itself is dependent on continued TGF-β/IL-6 or IL-23 signaling and is blocked by interferon-γ and IL-4 signaling. In response to IL-12 and IL-4, *in vitro* generated Th17 cells are converted into Th1 or Th2 cells, respectively. Th17 cells isolated *ex vivo*, however, maintain their IL-17 memory upon subsequent *in vitro* culture, even in the absence of IL-23. Their cytokine memory is not regulated by IL-12 or IL-4. Th17 cells generated *in vivo* are a stable and distinct lineage of Th cell differentiation.

Key words: Cytokine memory · Interleukin-17 · T-cell differentiation

Supporting Information available online

Introduction

Th memory lymphocytes are imprinted for the re-expression of distinct cytokine genes upon restimulation. Originally, two types of Th effector memory cells had been defined: T helper type 1 (Th1) cells re-expressing interferon-γ (IFN-γ), and Th2 cells, re-expressing interleukin (IL)-4, -5 and -13 [1]. Recently, a third lineage of Th effector memory cells has been described, characterized by the re-expression of IL-17A, IL-17F and IL-22 (reviewed in [2]). Th17 cells can induce autoimmune inflammation [3] and are protective in response to fungal infection [4]. *In vitro*, naïve murine Th cells can be induced to differentiate into Th17 cells by combined TGF-β and IL-6 signalling [5, 6]. IL-23 promotes survival and proliferation of Th17 cells [6]. IL-21 can induce IL-17 independent of IL-6 and is expressed by Th17 cells themselves, as part of a positive regulatory feedback loop for IL-17 re-expression [7, 8]. In human Th cells, similar signals are required for the differentiation of IL-17 re-expressing Th memory cells [9–11]. STAT3 is involved as a signal transducer and IRF-4 [12] and the retinoic acid receptor-related orphan

Correspondence: Dr. Hyun-Dong Chang
e-mail: chang@drfz.de

*These authors contributed equally to the work.

receptors RORγt [13] and RORα [14] as transcription factors controlling lineage development. Ectopic over-expression of RORγt and RORα in naïve Th cells is sufficient to induce IL-17 expression [14].

As part of their functional memory, the capacity of effector memory Th cells to stably re-express particular cytokines has been demonstrated for Th1 cells and IFN-γ expression and for Th2 cells and their IL-4 and IL-10 expression [15, 16]. This memory cytokine expression depends on TcR signals, but does not require the original instructive signals. It even occurs in the presence of adverse instructive signals. Cytokine memory for *Il4* and *Ifnγ* is based on epigenetic modification of the cytokine genes and expression of the transcription factors T-bet and GATA-3, for Th1 and Th2 differentiation, respectively (reviewed in [17]). A more complex situation has been described for the cytokine IL-10, which can be expressed by Th1 and Th2 cells. In Th2 cells, the *Il10* gene is imprinted by GATA-3, but this imprinting requires multiple restimulations of the Th2 cells, while the *Il4* gene is imprinted in the primary activation [18–20]. Th1 cells are imprinted to re-express *Il10* by Notch [21], but re-expression requires continued IL-12 signaling [18].

Chromatin of the *Il17a* and *Il17f* genes is modified in Th17 cells, as compared with Th1 and Th2 cells [22], and their re-expression depends on RORγt and RORα [14]. It is not clear, however, whether the chromatin-modifications and the ROR transcription factors imprint the *Il17* genes for re-expression, and whether Th17 cells induced with the signals identified so far are resistant to reprogramming by IL-12 or IL-4? *In vitro*, IL-4 and IFN-γ inhibit the induction of Th17 differentiation [23]. Interestingly, Th cells co-expressing IL-17 and IFN-γ are frequent *in vivo*, indicating that *in vivo* IFN-γ and IL-17 inductions are not mutually exclusive [4, 24–26].

To analyze the cytokine memory of individual Th cells expressing IL-17, we here have isolated Th cells according to the secretion of IL-17 and analyzed their cytokine memory upon further stimulation *in vitro*. IL-17-secreting cells generated *in vitro,* by TGF-β, IL-6 and IL-23 in the absence of IFN-γ and IL-4, upregulate the lineage-specific transcription factors RORγt and RORα but fail to re-express IL-17 in the absence of the original inducing signals, even after repeated instructive restimulation. They are converted to express IFN-γ when stimulated with IL-12, or IL-4 when stimulated in the presence of IL-4. In contrast, IL-17 expression could not be induced in differentiated Th1 and Th2 cells by TGF-β, IL-6 and IL-23, blocking IL-4 and IFN-γ. IL-17-expressing memory Th cells isolated *ex vivo* faithfully re-express IL-17, even when restimulated in the absence of IL-17-inducing signals or in the presence of IL-4 or IL-12.

Results

IL-17 is not induced in Th1 and Th2 memory cells

Naïve $CD4^+CD62L^+$ T cells from TCR transgenic DO11.10 mice were activated with their cognate antigen and differentiated into Th1 cells with IL-12 and anti-IL-4 antibody, or into Th2 cells with IL-4, anti-IL-12 and anti-IFNγ. After 6 days the Th1 and Th2 memory cells were restimulated with antigen, but this time under Th17 inducing conditions, *i.e*. in the presence of TGF-β, IL-6, IL-23, anti-IL-4 and anti-IFN-γ. Six days later, the cells were restimulated with PMA/ionomycin, fixed after 4 h, permeabilized and stained intracellularly for cytokine expression. Induction of IL-17 by TGF-β and IL-6 was not effective in either Th1 (1.6% $IL\text{-}17^+$ cells) or Th2 (4.7%) memory cells (Figs. 1A and B). When naïve Th cells were differentiated into Th17 cells for 6 days and then restimulated under Th17 polarizing conditions for an additional 6 days, IL-17 expression increased from 12.9 to 29% (Fig. 1C). To exclude inhibition of Th17 differentiation by IFN-γ in established Th1 cells, we also analyzed cells deficient for the IFN-γ receptor. Also in these cells, once the cells had been polarized into Th1 cells, IL-17 expression was not effectively induced (4%) (Supporting Information Fig. 1), as compared with naïve cells (25%) (Supporting Information Fig. 2). In Th1 cells, T-bet was upregulated under Th17-inducing conditions (Fig. 1D). The transcription factors RORα and RORγt were upregulated 2- and 6-fold, respectively, in Th1 cells under Th17-inducing conditions. In Th2 cells, GATA-3 was downregulated 2–3-fold when they had been restimulated under Th17-inducing conditions, and RORγt expression was upregulated 5-fold. However, RORγt levels remained well below the expression level in cells stimulated twice under Th17 polarizing conditions. The expression of IL23R and RORα remained unchanged.

Direct isolation of IL-17-expressing Th cells

To analyze the stability of Th17 memory cells on the single cell level, we developed a cytometric cytokine secretion assay [27, 28] for murine IL-17. Upon PMA/ionomycin restimulation the maximal frequency of IL-17-expressing Th cells is reached already after 1–2 h (Supporting Information Fig. 3). Accordingly, Th17 cells were restimulated for 1 h to induce cytokine expression, labeled with the IL-17 capture matrix, and allowed to secrete IL-17 for 30 min. The secreted IL-17 bound to the capture matrix was then detected by a fluorochrome-conjugated anti-IL-17 antibody. The cells were analyzed by flow cytometry and separated by fluorescent-activated cell sorting (FACS) (Fig. 2A) or magnetic cell sorting (data not shown). Cells placed on ice for the secretion period, thus blocking secretion, were used as control (Fig. 2B, left plot). The capacity of the capture matrix was determined by adding recombinant IL-17 (Fig. 2B, right plot). To control for false-positive cells due to cross-feeding of IL-17 from secreting cells to the capture matrix of non-secreting cells, cells of the IL-17 secretion assay were fixed and stained intracellularly for IL-17 in the presence or absence of the membrane-permeabilizing agent saponin. All IL-17-secreting but none of the IL-17-non-secreting Th cells expressed intracellular IL-17 (Fig. 2C).

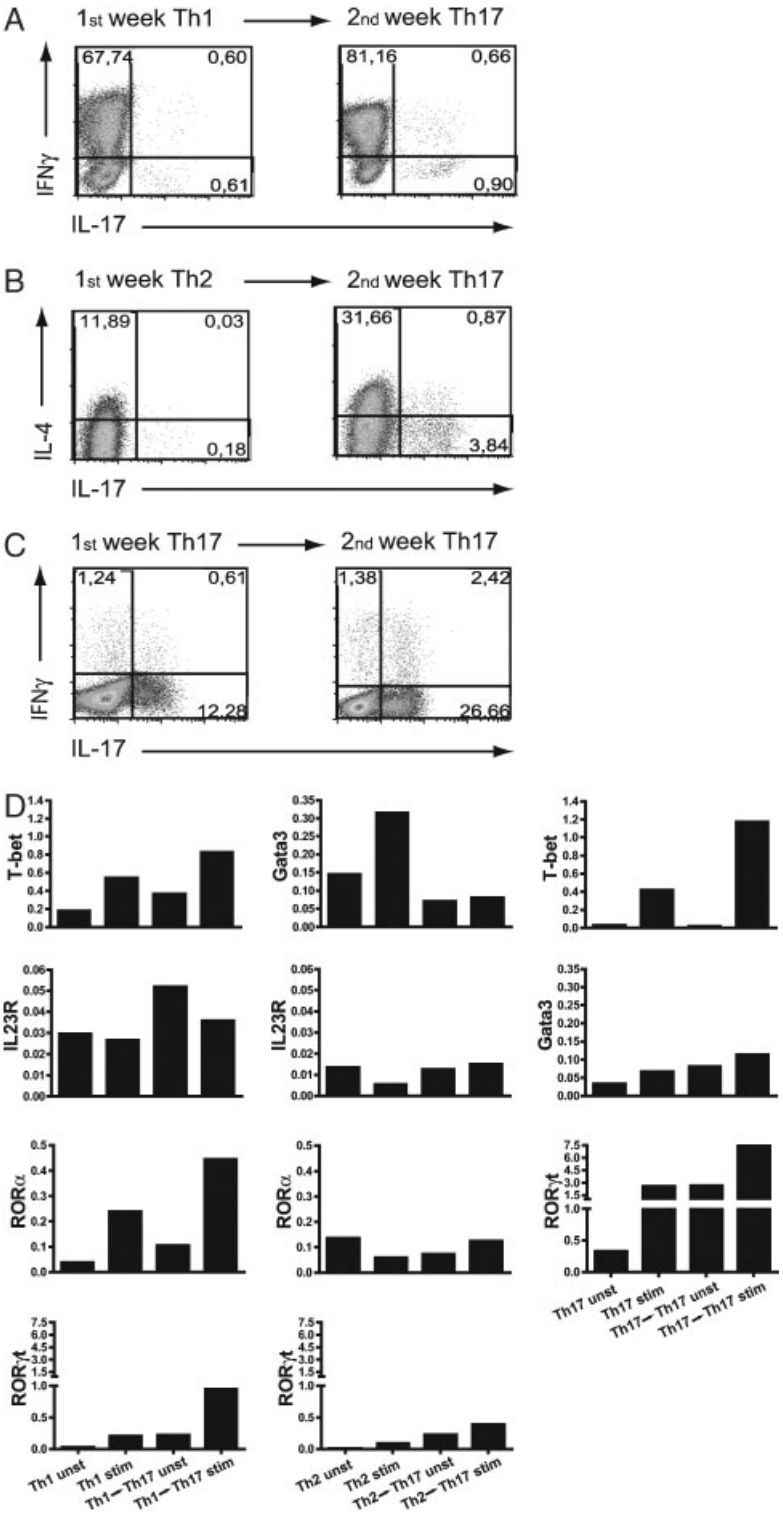


Figure 1. Th1 and Th2 cells are refractory to Th17 polarization. Naïve $CD4^+CD62L^{high}$ cells from DO11.10 mice were differentiated in the presence of rIL-12, anti-IL-4, irradiated APC and $OVA_{323-339}$ to Th1 (A), in the presence of IL-4, anti-IL-12, anti-IFN-γ, irradiated APC and $OVA_{323-339}$ to Th2 (B), or in the presence of TGF-β, IL-6, IL-23, anti-IFN-γ, anti-IL-4, irradiated APC and $OVA_{323-339}$ to Th17 (C) for 6 days. The cells were cultured for another 6 days in the presence of IL-17-inducing conditions (TGF-β, IL-6, IL-23, anti-IL-4 and anti-IFN-γ). Cytokine expression was analyzed after 5 h of restimulation with PMA/ionomycin by intracellular cytokine staining. Data are representative of four experiments. (D) RNA was extracted from unstimulated (unst) cells and stimulated cells (stim) for 3 h and quantitative real-time PCR was performed for T-bet, GATA-3, IL-23R, RORα and RORγt. Data are representative of two experiments.

IL-17 re-expression is blocked by IL-4 and IFN-γ

We stimulated naïve $CD4^+CD62L^+$ cells from DO11.10 mice with antigen in the presence of TGF-β, IL-6, IL-23, anti-IL-4 and anti-IFN-γ to induce Th17 differentiation. After 6 days, such cells expressed IL-17, IL-22, IL-17F, IL-23 receptor, RORγt and RORα (Supporting Information Fig. 2). Cells from such cultures were separated into IL-17 expressing and non-expressing cells, with a purity of >97 and >99%, respectively (Fig. 3A). In either population, we could not detect IFN-γ- or IL-4-expressing cells

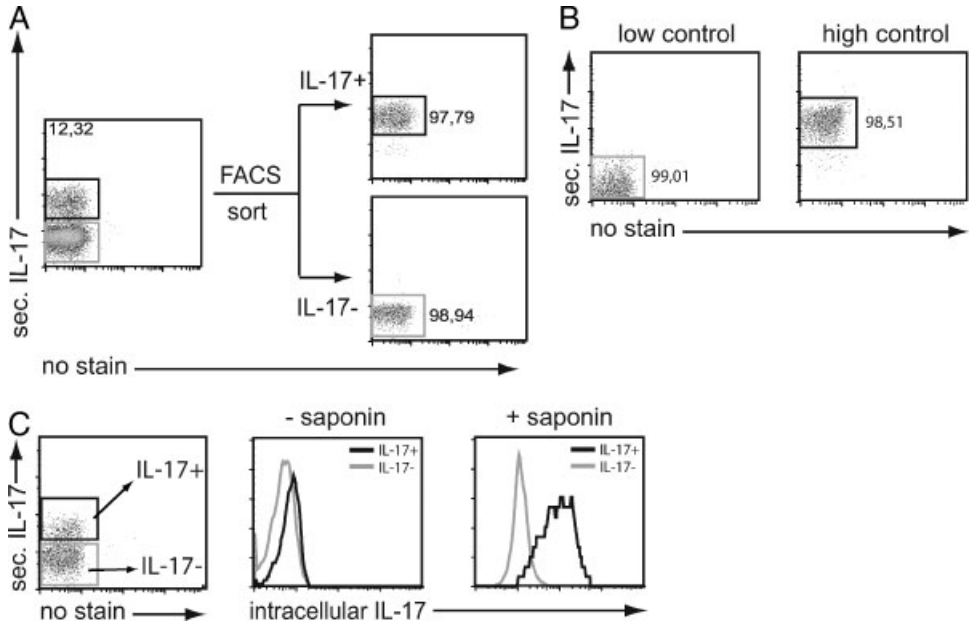


Figure 2. Isolation of viable IL-17 producing and non-producing cells with the IL-17 secretion assay. (A) Naïve $CD4^+CD62L^{high}$ cells were differentiated under IL-17-inducing conditions for 6 days, and the IL-17 secretion assay was performed. IL-17 secreting and IL-17 non-secreting cells were separated by FACS sorting. (B) After labeling with the IL-17 capture matrix, cells were either kept on ice immediately to prevent secretion (low control) or incubated with recombinant murine IL-17 at RT for 15 min (high control). (C) The staining of secreted IL-17 correlates with the staining of intracellular IL-17. After the IL-17 secretion assay, the cells were fixed and stained for intracellular IL-17. To confirm the staining of intracellular IL-17, the staining was performed either in the absence or in the presence of saponin. Data are representative of two experiments.

(data not shown). $IL\text{-}17^+$ and $IL\text{-}17^-$ cells were restimulated with the cognate antigen, cultured for another 6 days under various conditions, and analyzed for IL-17 re-expression. We did not observe selective outgrowth of contaminating cells in either purified population, using CFSE to track proliferation of the cells (Supporting Information Fig. 4). Cell numbers were comparable and viability was above 90% throughout the culture period, as monitored microscopically (data not shown). When cultured in the absence of exogenous cytokines and blocking antibodies, only 13% of the $IL\text{-}17^+$ Th cells re-expressed IL-17, 49% now expressed IFN-γ (Fig. 3A). In the presence of IL-23, 32% of the $IL\text{-}17^+$ cells re-expressed IL-17, and 16% of them expressed IFN-γ. In the presence of blocking antibodies to IL-4 and IFN-γ, more than 60% of the $IL\text{-}17^+$ cells re-expressed IL-17, irrespective of whether IL-23 was blocked by anti-IL-12p40, or added. This frequency was also not influenced by addition of recombinant TGF-β and IL-6. IL-17 non-expressing cells expressed IFN-γ (35%) but no IL-17 (<1%), if no cytokines or antibodies were added. In the presence of IL-23, 2% of these cells expressed IL-17. Blocking of IFN-γ and IL-4 resulted in the expression of IL-17 in 9% of the cells in the absence of IL-23, 12% in the presence of IL-23 and 18% in the presence of TGF-β, IL-6 and IL-23 (Fig. 3A). In $IL\text{-}17^+$ and $IL\text{-}17^-$ cells, RORγt and RORα were expressed at similar levels (Fig. 3B) and were downregulated when the cells were cultured without the addition of exogenous antibodies or cytokines. Regulation of RORγt expression corresponded with the expression of IL-17, being high under conditions when IL-17 was expressed (anti-IL-4 and anti-IFNγ with anti-IL-12p40, IL-23 or TGF-β/IL-6). RORα was generally downregulated upon reculture, except for a 4-fold upregulation in the presence of TGF-β/IL-6 compared with cells cultured just in the presence of anti-IL-4, anti-IFN-γ and IL-23. T-bet expression in $IL\text{-}17^+$ cells was 3-fold enhanced in the presence of (endogenous) IFN-γ. The expression of T-bet was higher in $IL\text{-}17^-$ than in $IL\text{-}17^+$ cells. IL-17F, IL-22, IL-23R and IL-21 were only highly expressed by $IL\text{-}17^+$ sorted cells, and their expression was downregulated in the absence of added cytokines or antibodies. IL-23 receptor expression was maintained in the presence of IL-23, but downregulated in the presence of TGF-β and IL-6. IL-17F expression was only maintained in the presence of TGF-β, IL-6 and IL-23. IL-22 and IL-21 expression was downregulated under all conditions analyzed (Fig. 3B).

Th17 cells generated *in vitro* can cross-differentiate into Th1 and Th2 cells

We stimulated naïve $CD4^+$ Th cells with TGF-β, IL-6, IL-23, anti-IL-4 and anti-IFN-γ for 6 days and isolated IL-17 expressing and non-expressing cells. $IL\text{-}17^+$ and $IL\text{-}17^-$ cells were restimulated and cultured for an additional 6 days either under Th1 polarizing conditions (IL-12 and anti-IL-4) or under Th2 polarizing conditions (IL-4, anti-IFNγ and anti-IL-12). As shown in Fig. 4A, $IL\text{-}17^+$ cells re-expressed IL-17 with a frequency of 8% under Th1 polarizing conditions, and 26% under Th2 polarizing

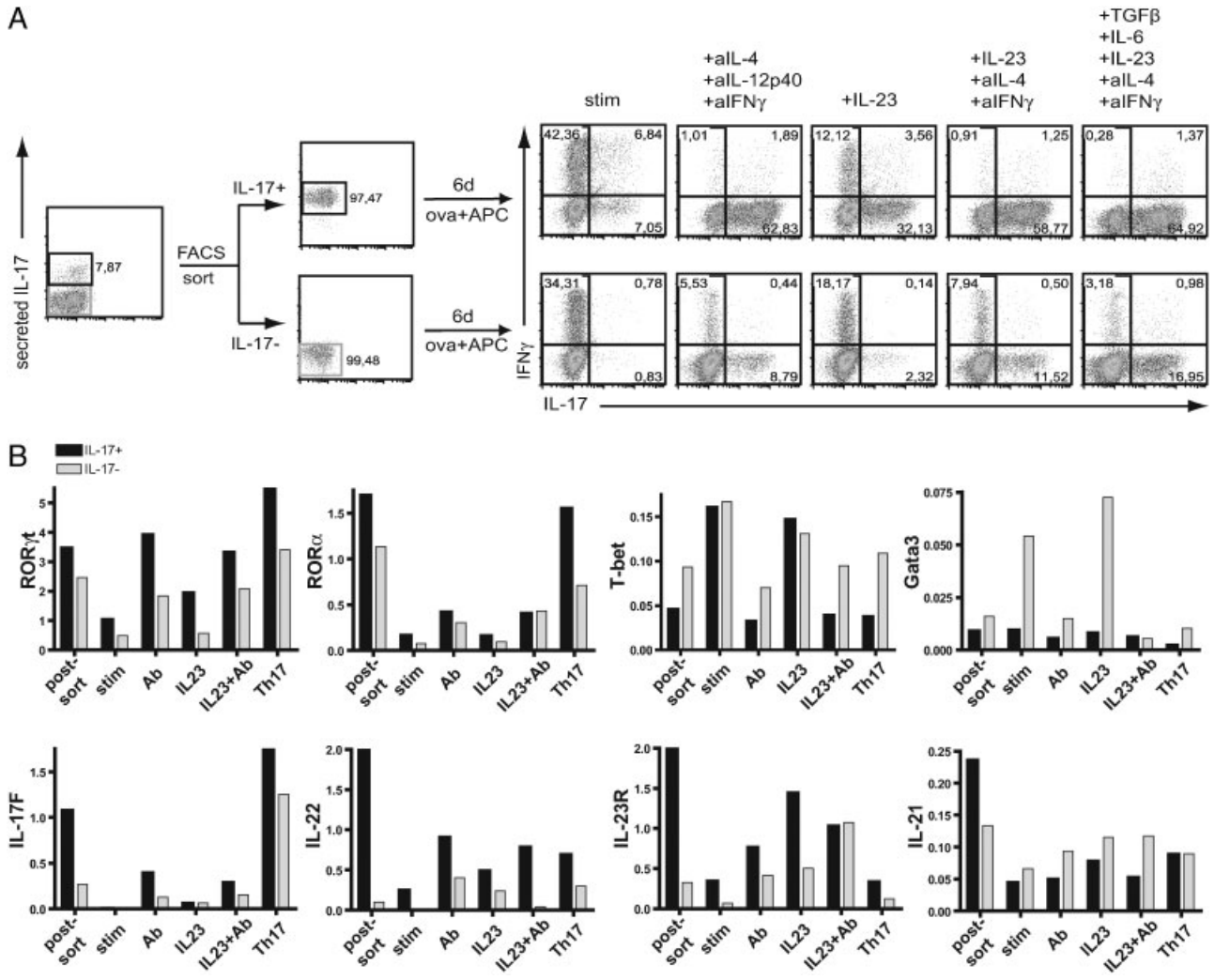


Figure 3. *In vitro*-generated Th17 cells fail to re-express IL-17. Naïve $CD4^+CD62L^{high}$ cells from DO11.10 mice were differentiated under IL-17 inducing conditions for 6 days. (A) IL-17 producing and non-producing cells were separated and recultured for 6 days in the presence of $OVA_{323-339}$ and irradiated APC only (stim), under neutral conditions (anti-IL-4, anti-IL-12, anti-IFN-γ) (Ab) or under different Th17 favoring conditions (IL-23, IL-23 with anti-IL-4 and anti-IFN-γ (IL23+Ab) or TGF-β, IL-6, IL-23, anti-IL-4 and anti-IFN-γ (Th17)). Cytokine expression was analyzed by intracellular staining after PMA/ionomycin for 5 h. Data are representative of three experiments. (B) mRNA was extracted from $IL\text{-}17^+$ and $IL\text{-}17^-$ cells directly after isolation (post-sort) and 6 days later from cells restimulated for 2 h, reverse transcribed and quantified by quantitative real-time PCR for RORγt, RORα, T-bet, GATA-3, IL-17F, IL-22, IL-23R and IL-21. Data are representative of two experiments.

conditions. $IL\text{-}17^+$ and $IL\text{-}17^-$ cells started to express IFN-γ (>70% of $CD4^+$ cells) or IL-4 (19% in $IL\text{-}17^+$ and 28% in $IL\text{-}17^-$ cultures), respectively. RORγt, RORα, IL-17F, IL-22, IL-23R and IL-21 expression was downregulated when the cells were cultured under Th1 or Th2 conditions. Under Th1 conditions the cells upregulated the expression of T-bet 3–8-fold. Under Th2 conditions GATA-3 expression was upregulated 16–20-fold (Fig. 4C). The memory for IL-17 re-expression was also not stabilized in cells polarized toward Th17 differentiation for 3 wk, with weekly restimulations (Fig. 4B). After 3 wk, $IL\text{-}17^+$ cells were isolated and further cultured for 6 days under Th1 or Th2 polarizing conditions. Only 9% re-expressed IL-17 under such conditions. About 64% of the $IL\text{-}17^+$ or $IL\text{-}17^-$ cells expressed IFN-γ and 12–13% expressed IL-4 under Th1 or Th2 conditions, respectively.

In vivo-generated Th17 cells maintain IL-17 expression *in vitro*

IL-17-expressing Th memory cells generated *in vivo* were isolated directly *ex vivo* from unmanipulated DO11.10 or BALB/c mice (Fig. 5, Supporting Information Fig. 5). $CD4^+CD62L^{low}$ splenocytes were stimulated polyclonally with PMA/ionomycin and IL-17-expressing cells isolated with the IL-17 secretion assay. Of the PMA-ionomycin-stimulated cells, 8.2% expressed IFN-γ, 0.2% IL-4, and 3.6% expressed IL-17, of which approx. 25% co-expressed IFN-γ (Fig. 5A). Purified $IL\text{-}17^+$ and $IL\text{-}17^-$ cells were recultured for 6 days *in vitro*, either without adding or blocking cytokines, or adding IL-23, or under Th1 or Th2 polarizing conditions (Fig. 5B). In the absence of added antibodies or cytokines 72% of the $IL\text{-}17^+$ cells re-expressed

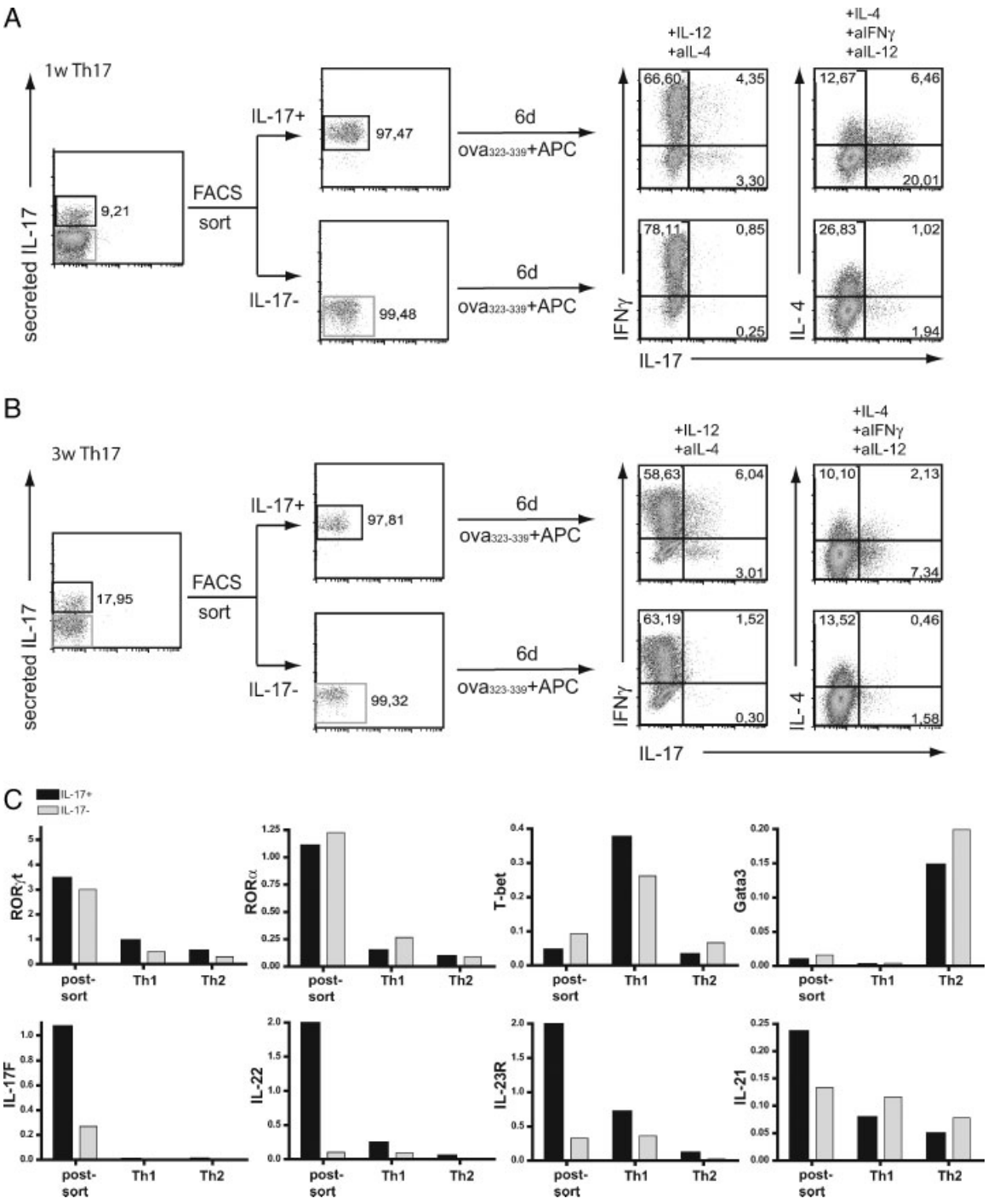


Figure 4. *In vitro* generated-Th17 cells can be converted to Th1 and Th2 cells. Naïve $CD4^+CD62L^{high}$ cells from DO11.10 mice were differentiated under IL-17 inducing conditions. (A) After 6 days, the cells were restimulated for IL-17 secretion assay. The $IL\text{-}17^+$ and $IL\text{-}17^-$ cells were cultured under Th1 (IL-12, anti-IL-4) and Th2 (IL-4, anti-IL-12, anti-IFN-γ) polarizing conditions for 6 days. Cytokine expression was analyzed by intracellular staining after PMA/ionomycin restimulation. (B) Cells were cultured for 18 days under Th17 polarizing conditions. $IL\text{-}17^+$ and $IL\text{-}17^-$ cells were separated and cultured under Th1 and Th2 polarizing conditions. Data are representative of three experiments. (C) mRNA of 1-week-old $IL\text{-}17^+$ and $IL\text{-}17^-$ cells directly after isolation and after 6 day culture under Th1 and Th2 polarizing conditions was isolated after 2 h restimulation with PMA/ionomycin, reverse transcribed and quantified by quantitative real-time PCR for RORγt, RORα, T-bet, GATA-3, IL-17F, IL-22, IL-23R and IL-21. Data are representative of two experiments.

IL-17. In the presence of added IL-23, 83% re-expressed IL-17. *Ex vivo*-isolated $IL\text{-}17^+$ Th cells were refractory to Th1 and Th2 polarizing signals. Under Th1 conditions the frequency of IFN-γ-expressing cells was 14%. Under Th2 polarizing conditions, 4% of IL-4-expressing cells were observed. About 75 and 68% of the $IL\text{-}17^+$ cells re-expressed IL-17 under Th1 and Th2 conditions,

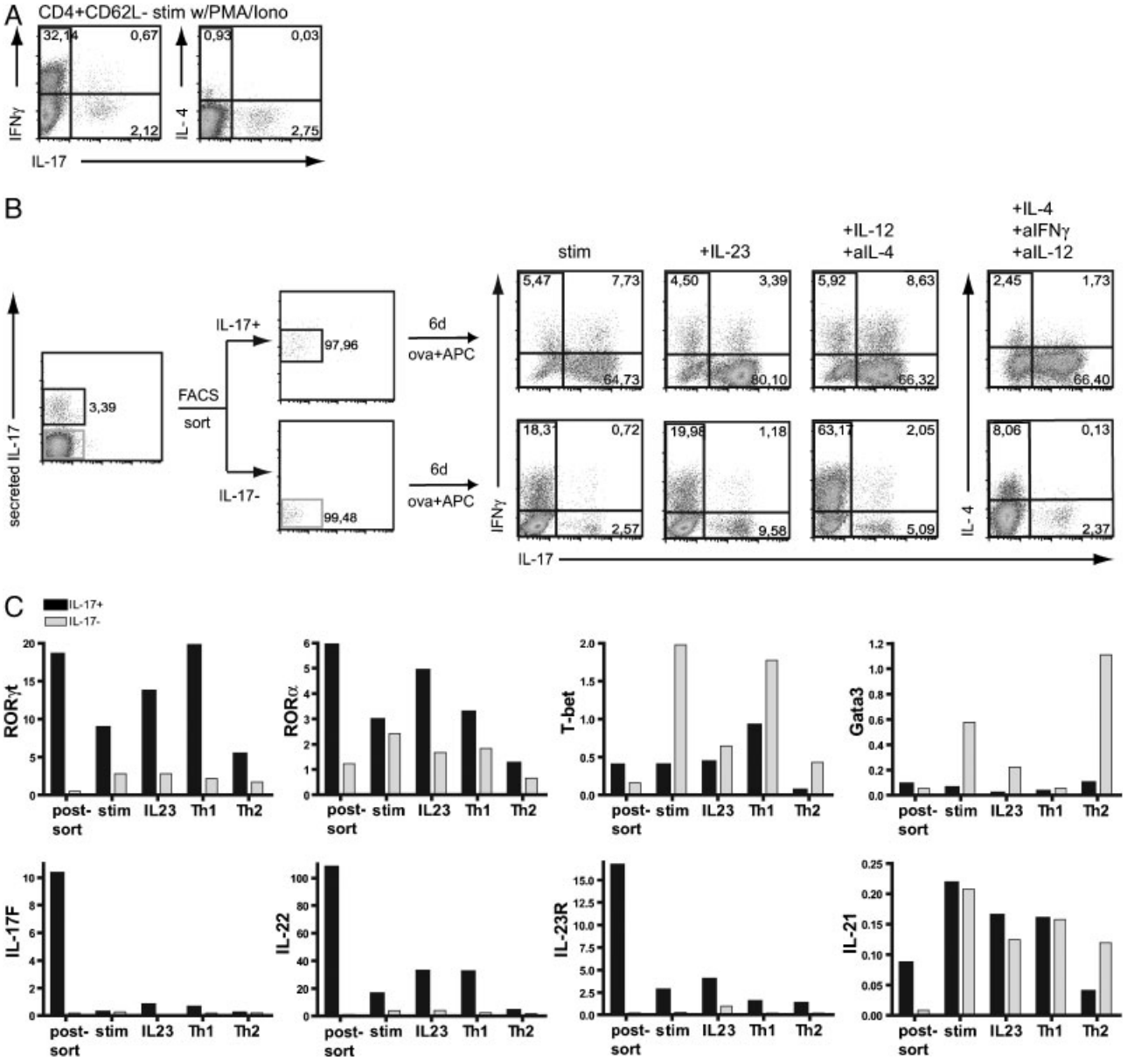


Figure 5. *In vivo*-generated Th17 cells have a stable memory for IL-17. Memory $CD4^+CD62L^{low}$ cells from 6-months-old DO11.10 mice were isolated and either restimulated for (A) direct cytokine analysis or (B) IL-17 secretion assay. The IL-17^+ and IL-17^- cells were cultured without (stim) or with IL-23 and under Th1- or Th2-polarizing conditions for 6 days. The cytokine expression was analyzed by intracellular staining after restimulation with PMA/Ionomycin for 5 h. Data are representative of three experiments. (C) mRNA of *ex vivo*-isolated IL-17^+ and IL-17^- cells directly after isolation and cells cultured for 6 days was isolated after 2 h restimulation with PMA/ionomycin, reverse transcribed and quantified by quantitative real-time PCR. Data are representative of two experiments.

respectively. The expression of RORγt and RORα was downregulated 5-fold under Th2 conditions. GATA-3 expression was not induced in IL-17^+ cells under any condition. The expression of T-bet was upregulated 2-fold under Th1-inducing conditions in the IL-17^+ cells. In IL-17^- cells, RORγt and RORα were not upregulated. T-bet and GATA-3 were induced under neutral and Th1 or Th2 conditions. IL-23 receptor, IL-22 and IL-17F were highly expressed in IL-17^+ cells and their expression was downregulated upon *in vitro* culture. IL-21 expression was upregulated at least 2-fold upon *in vitro* culture, except when cultured under Th2 conditions, which led to a 2-fold reduction in IL-21 expression (Fig. 5C).

Discussion

The expression of IL-17 by Th lymphocytes has been originally described many years ago [24, 29]. Recently IL-17-expressing Th lymphocytes have been recognized as a separate lineage of Th cell differentiation, distinct from the Th1 and Th2 lineages [23].

Stability and plasticity of the cytokine memory of Th17 memory effector cells has been a matter of debate, in particular in light of reports of Th cells expressing both IL-17 and IFN-γ [4, 24–26].

Here we describe a cytometric cytokine secretion assay for murine IL-17 and its use to analyze the memory of IL-17-expressing Th cells for expression of IL-17. Isolated IL-17$^+$ or IL-17$^-$ Th cells showed the same proliferation and survival upon reculture. IL-17-expressing cells generated *in vitro* by stimulation of activated naïve Th cells with TGF-β, IL-6 and IL-23, and blocking of IFN-γ and IL-4 with anti-IFNγ and anti-IL-4, failed to re-express IL-17 upon later reactivation, when the original inducing signals were lacking, or IFN-γ and IL-4 were not neutralized. IL-17$^+$ Th cells, even after 3 wk of repeated instruction for IL-17 expression could still be converted into IFN-γ-expressing Th1 cells with IL-12 or into IL-4-expressing Th2 cells with IL-4. In contrast to *in vitro*-generated Th17 cells, IL-17-expressing Th cells isolated *ex vivo* maintained a memory for IL-17 expression *in vitro*, even in the presence of IL-12 or IL-4.

For the cytokine memory of Th1 and Th2 cells, molecular mechanisms have been described, which prevent the differentiation of Th1 into Th2 cells and *vice versa*. Such mechanisms include the mutual inhibition of the master transcription factors T-bet and GATA-3 [30], the downregulation of receptors for costimulatory signals [31] and the epigenetic silencing of cytokine genes (reviewed in [32]). Here we show that Th1 and Th2 effector memory cells also cannot be converted into Th17 cells, at least not by TGF-β, IL-6 and IL-23 signals, blocking IL-4 and IFN-γ. Interestingly, in Th1 cells, under Th17 polarizing conditions RORα and RORγt were upregulated 2- and 6-fold, respectively. Apparently this upregulation of Th17 lineage master transcription factors is not sufficient for the induction of IL-17 expression in such Th1 cells. This may be due to even further upregulation of T-bet in Th1 cells under Th17 polarizing conditions. T-bet has been described as a negative regulator of Th17 differentiation [33, 34]. In Th2 cells, GATA-3 is downregulated 2–3-fold and RORγt upregulated 4-fold upon restimulation in a Th17 inducing cytokine milieu. The expression of IL-23 receptor and RORα was not upregulated. In apparent contradiction to our results, it has been shown that ectopic expression of RORγt in combination with RORα in Th1 and Th2 cells can lead to expression of IL-17 [14]. However, RORγt and RORα in those experiments were expressed in Th cells perhaps not fully committed to the Th1 or Th2 lineage. Future analysis of the *Il17* gene with respect to epigenetic silencing in Th1 and Th2 cells will clarify whether exclusion of IL-17 expression in Th1 and Th2 cells is analogous to the reciprocal silencing of the *Il4* gene in Th1 or the *Ifnγ* gene in Th2 cells [35, 36]. The methylation of single CpG sites in cytokine gene promoters has been shown to silence gene expression by preventing binding for TcR responsive transcription factors [36, 37].

The expression of IL-17F in the *in vitro*-generated as well as in the *ex vivo*-isolated Th17 cells did not correlate with the re-expression of IL-17 after reculture. IL-17F is highly homologous to IL-17 and the *Il17f* gene is adjacent to the *Il17* gene [38], suggesting a coordinated expression. While being highly expressed initially in cells sorted for IL-17 expression, IL-17F expression was only maintained in the presence of TGF-β and IL-6 (Fig. 3B). Interestingly, IL-17F expression was not stable in *ex vivo*-isolated IL-17-expressing cells, either, indicating that the memory for IL-17F re-expression is conditional and depends on different or additional signals than that for IL-17 re-expression, and that maintained RORγt and RORα expression may be required [14] but not be sufficient for IL-17F expression.

Our results suggest that the currently available *in vitro* protocols for the induction of IL-17 expression in naïve Th lymphocytes lack signals for the induction of a stable cytokine memory for IL-17 re-expression. Although IL-17 expression was induced very efficiently *in vitro* by TGF-β, IL-6, IL-23 and anti-IFNγ and anti-IL-4, failure to block IFN-γ or IL-4 during subsequent restimulation and culture led to the loss of IL-17 re-expression in cells which once had expressed IL-17. Both IFN-γ and IL-4 had been described as negative regulators of IL-17 expression [23, 39]. In *in vitro*-generated Th17 cells, the expression of RORγt and RORα was downregulated in conditions under which the cells did not re-express IL-17 (Fig. 3B) and in particular under Th1 or Th2 polarizing conditions (Fig. 4B). Unlike the memory for IL-10 expression in Th2 cells, which requires multiple rounds of stimulation by IL-4 for stability [18, 19], repeated *in vitro* stimulation in the presence of TGF-β, IL-6 and IL-23 did not lead to a stable commitment for IL-17 expression (Fig. 4B). Such cells were still plastic and responded to the presence of IL-12 or IL-4 with differentiation into IFN-γ-expressing Th1 or into IL-4-expressing Th2 cells, respectively. It remains to be shown how the functional imprinting of *in vivo*-generated Th17 cells is encoded on the molecular level, to what extent the transcription factors RORγt and RORα, STAT-3 and IRF-4 [12–14, 40] are involved in the imprinting of the *Il17* locus and whether their genes themselves are imprinted.

Our results are in apparent contrast to previously published data of Harrington *et al.* [23] and Park *et al.* [39] with regard to the apparent stability of *in vitro*-generated Th17 cells when stimulated with IFN-γ or IL-4. In both reports, the authors generated Th17 cells from bulk CD4$^+$ (Harrington *et al.* [23]) or CD4$^+$CD62L$^-$ "memory" Th cells (Park *et al.* [39]) from spleen and lymph nodes, not from purified naïve Th cells. Th17 cells were then "generated" by IL-23. As we know now, through the work of Veldhoen *et al.* [6], IL-23 does not induce naïve Th cells to become Th17 cells, but rather expands pre-existing Th17 cells. In view of the work of Veldhoen *et al.* [6] and in view of our present data, the data of Harrington *et al.* [23] and Park *et al.* [39] have to be reinterpreted as showing the stability of *in vivo*-generated Th17 memory cells, and their selective expansion by IL-23 *in vitro*.

We here have demonstrated that IL-17-expressing Th cells generated *in vivo* are a stable lineage of effector memory cells, distinct from Th1 and Th2 cells, and functionally imprinted for re-expression of IL-17 upon TCR stimulation, even in the presence of Th1- or Th2-inducing conditions. IL-17-expressing cells generated *in vitro* are not functionally imprinted for IL-17 re-expression. Their re-expression of IL-17 depends on the continued presence of the canonical Th17-inducing signals.

Materials and methods

Mice

BALB/c and OVA-TCRtg/tg DO11.10 mice (kind gift of Dennis Y. Loh and Kenneth Murphy, Washington University School of Medicine, St. Louis, MO) were bred under specific pathogen-free conditions in our animal facility. IFN-$\gamma R^{-/-}$ mice were kindly provided by Thomas Schüler (Charité, Campus Benjamin Franklin, Berlin, Germany). The mice were sacrificed by cervical dislocation. All animal experiments were performed in accordance with institutional, state and federal guidelines.

Antibodies

All antibodies used in these experiments were either conjugated in-house or purchased as indicated. Anti-IL-4 (11B11), anti-IL-12 (C17.18), anti-IFN-γ(AN17.18.24) antibodies were purified from hybridoma supernatants at the German Rheumatism Research Center and used at 10 μg/mL final concentration. FITC-conjugated anti-CD4 (GK1.5), PE-conjugated anti-IL-17 (TC11-18H10; BD Pharmingen, San Diego, CA) and Cy5-conjugated anti-IL-4 (11B11) were used for all intracellular cytokine stainings.

Cell culture conditions and Th differentiation *in vitro*

$CD4^+CD62L^+$ cells from 6–8-wks-old OVA-$TCR^{tg/tg}$ DO11.10 mice were isolated as described previously [15]. All cultures and assays were carried out in RPMI supplemented with 10% FBS (Sigma Chemicals, St. Louis, MO), 100 U/mL of penicillin, 0.1 mg/mL of streptomycin, 0.3 mg/mL of glutamine (Invitrogen) and 10 μM β-mercaptoethanol at 37°C in 5% CO_2. Cell cultures were set up at 3×10^6 cells/mL and stimulated in the presence of 0.5 mM cognate peptide $OVA_{323–339}$ with irradiated (30 Gy) splenocytes isolated from BALB/c mice as APC. For Th1 differentiation, cells were stimulated in the presence of recombinant IL-12 (5 ng/mL; R&D Systems, Minneapolis, MN) and anti-IL-4 (11B11) antibody for 6 days. For Th2 differentiation, cells were stimulated in the presence of IL-4 (100 ng/mL, culture supernatant of HEK293 T cells transfected with murine IL-4 cDNA), anti-IL-12 (C17.8) and anti-IFN-γ(AN18.17.24) antibodies. For Th17 differentiation, cells were stimulated in the presence of TGF-β1 (1 ng/mL), IL-6, IL-23 (20 ng/mL) (all from R&D Systems), anti-IL-4 and anti-IFN-γ.

Isolation of IL-17-secreting cells *in vitro* and *ex vivo*

Cells were cultured under IL-17-inducing conditions for 6 days, or $CD4^+CD62L^{low}$ cells were isolated from spleen of 6-months-old DO11.10 or BALB/c mice. Cells were harvested and restimulated with 10 ng/mL of PMA (Sigma Chemicals) and 1 μg/mL of ionomycin (Sigma Chemicals) for 1.5 h. The cells were washed twice in ice-cold PBS with 0.5% w/v BSA (PBS/BSA). Cells were labeled for 5 min at 4°C with an IL-17-specific high-affinity capture matrix, *i.e.*, bi-specific Ab–Ab conjugates of an anti-CD45 antibody with an anti-IL-17 antibody (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Cell samples were taken for low control (kept on ice) and high control (incubated with recombinant IL-17 (0.5 μg/mL; Peprotech, Hamburg, Germany), washed after 10 min and kept on ice. The rest of the cells were transferred into 37°C warm RPMI medium at a low density (10^5 cells/mL) and placed at 37°C. Every 5 min the cells were mixed gently. After 30 min, the cells were transferred into ice-cold PBS/BSA and kept on ice for 10 min. The captured IL-17 was detected with an anti-IL17 biotin conjugated antibody followed by staining with an APC-conjugated anti-biotin antibody (Miltenyi Biotec). The IL-17 producing cells and the IL-17 non-producing cells were separated by FACSAria™ cell sorter (BD Biosciences). After sorting, the purity of the sort was confirmed with a FACSCalibur (BD Biosciences). Specificity of the IL-17 secretion assay was confirmed by intracellular staining.

Flow cytometry

A total of 4×10^6 cells/ml were stimulated in RPMI medium with 10 ng/mL of PMA and 1 μg/mL of ionomycin. An aliquot of 5 μg/mL Brefeldin A (Sigma Chemicals) was added after 1 h. After 5 h of stimulation, the cells were washed with PBS and fixed in 2% formaldehyde in PBS for 15 min at room temperature. The cells were stained for intracellular cytokines, as described previously [41]. FACS analysis was performed with a FACSCalibur, using CellQuest (BD Biosciences) and FlowJo (TreeStar, Ashland, OR) software.

RNA quantification

RNA preparation and cDNA synthesis was performed as previously described [18]. The expression of each gene was normalized to the expression of HPRT. Primer sets for the real-time PCR were as follows: HPRT up: 5′- GCT GGT GAA AAG GAC CTC T-3′, HPRT rev: 5′- CAC AGG ACT AGA CCT GC -3′; RORγ t up: 5′-TGC AAG ACT CAT CGA CAA GG -3′, RORγ trev: 5′- AGG GGA TTC AAC ATC AGT GC -3′; IL-17 for: 5′- TCC AGA AGG CCC TCA GAC TA -3′, IL-17 rev: 5′-AGC ATC TTC TCG ACC CTG AA -3′; IL-17F for: 5′- CAA AAC CAG GGC ATT TCT GT -3′, IL-17F rev: 5′- ATG GTG CTG TCT TCC TGA CC -3′; IL-22 for: 5′- GTC AAC CGC ACC TTT ATG CT -3′, IL-22 rev: 5′- CAT GTA GGG CTG GAA CCT GT -3′; IL-21for: 5′- ATC CTG AAC TTC TAT CAG CTC CAC -3′, IL-21 rev: 5′- GCA TTT AGC TAT GTG CTT CTG TTT C -3′; IL-21R for: 5′- TGT CAA TGT GAC GGA CCA GT -3′, IL-21R rev: 5′- CAC GTA GTT GGA GGG TTC GT -3′; RORα for: 5′- CCC CTA CTG TTC CTT CAC CA -3′, RORα rev: 5′- AGC TGC CAC ATC ACC TCT CT -3′; IL-23R for: 5′- AAC ATG ACA TGC ACC TGG AA -3′, IL-23R rev: 5′-TCC ATG CCT AGG GAA TTG AC-3′; Gata3 for: 5′-CCT ACC GGG TTC GGA TGT AAG T-3′, Gata3 rev:

5′-AGT TCG CGC AGG ATG TCC-3′; Tbet for: 5′-TCC TGC AGT CTC TCC ACA AGT -3′ and Tbet rev: 5′CAG CTG AGT GAT CTC TGC GT -3′.

Acknowledgements: This work has been supported by the German Science Foundation (DFG SFB421).

Conflict of interest: The authors declare no financial or commercial conflict of interest.

References

1 **Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A., and Coffman, R. L.,** Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* 1986. **136**: 2348–2357.

2 **Bettelli, E., Korn, T., and Kuchroo, V. K.,** Th17: the third member of the effector T cell trilogy. *Curr. Opin. Immunol.* 2007. **19**: 652–657.

3 **Langrish, C. L., Chen, Y., Blumenschein, W. M., Mattson, J., Basham, B., Sedgwick, J. D., McClanahan, T. *et al.*,** IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J. Exp. Med.* 2005. **201**: 233–240.

4 **Acosta-Rodriguez, E. V., Rivino, L., Geginat, J., Jarrossay, D., Gattorno, M., Lanzavecchia, A., Sallusto, F. and Napolitani, G.,** Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat. Immunol.* 2007. **8**: 639–646.

5 **Bettelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T. B., Oukka, M., Weiner, H. L. and Kuchroo, V. K.,** Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 2006. **441**: 235–238.

6 **Veldhoen, M., Hocking, R. J., Atkins, C. J., Locksley, R. M. and Stockinger, B.,** TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 2006. **24**: 179–189.

7 **Korn, T., Bettelli, E., Gao, W., Awasthi, A., Jager, A., Strom, T. B., Oukka, M. and Kuchroo, V. K.,** IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. *Nature* 2007. **448**: 484–487.

8 **Nurieva, R., Yang, X. O., Martinez, G., Zhang, Y., Panopoulos, A. D., Ma, L., Schluns, K. *et al.*,** Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature* 2007. **448**: 480–483.

9 **Volpe, E., Servant, N., Zollinger, R., Bogiatzi, S. I., Hupe, P., Barillot, E. and Soumelis, V.,** A critical function for transforming growth factor-[beta], interleukin 23 and proinflammatory cytokines in driving and modulating human TH-17 responses. *Nat. Immunol.* 2008. **9**: 650–657.

10 **Manel, N., Unutmaz, D. and Littman, D. R.,** The differentiation of human TH-17 cells requires transforming growth factor-[beta] and induction of the nuclear receptor ROR[gamma]t. *Nat. Immunol.* 2008. **9**: 641–649.

11 **Yang, L., Anderson, D. E., Baecher-Allan, C., Hastings, W. D., Bettelli, E., Oukka, M., Kuchroo, V. K. and Hafler, D. A.,** IL-21 and TGF-[beta] are required for differentiation of human TH17 cells. *Nature* 2008. **454**: 350–352.

12 **Brustle, A., Heink, S., Huber, M., Rosenplanter, C., Stadelmann, C., Yu, P., Arpaia, E. *et al.*,** The development of inflammatory T(H)-17 cells requires interferon-regulatory factor 4. *Nat. Immunol.* 2007. **8**: 958–966.

13 **Ivanov, I. I., McKenzie, B. S., Zhou, L., Tadokoro, C. E., Lepelley, A., Lafaille, J. J., Cua D. J. and Littman, D. R.,** The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+T helper cells. *Cell* 2006. **126**: 1121–1133.

14 **Yang, X. O., Pappu, B. P., Nurieva, R., Akimzhanov, A., Kang, H. S., Chung, Y., Ma, L. *et al.*,** T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. *Immunity* 2008. **28**: 29–39.

15 **Richter, A., Löhning, M. and Radbruch, A.,** Instruction for cytokine expression in T helper lymphocytes in relation to proliferation and cell cycle progression. *J. Exp. Med* 1999. **190**: 1439–1450.

16 **Tykocinski, L. O., Hajkova, P., Chang, H. D., Stamm, T., Sozeri, O., Lohning, M., Hu-Li J. *et al.*,** A critical control element for interleukin-4 memory expression in T helper lymphocytes. *J. Biol. Chem.* 2005. **280**: 28177–28185.

17 **Löhning, M., Richter, A. and Radbruch, A.,** Cytokine memory of T helper lymphocytes. *Adv. Immunol.* 2002. **80**: 115–181.

18 **Chang, H. D., Helbig, C., Tykocinski, L., Kreher, S., Koeck, J., Niesner, U. and Radbruch, A.,** Expression of IL-10 in Th memory lymphocytes is conditional on IL-12 or IL-4, unless the IL-10 gene is imprinted by GATA-3. *Eur. J. Immunol.* 2007. **37**: 807–817.

19 **Lohning, M., Richter, A., Stamm, T., Hu-Li, J., Assenmacher, M., Paul, W. E. and Radbruch, A.,** Establishment of memory for IL-10 expression in developing T helper 2 cells requires repetitive IL-4 costimulation and does not impair proliferation. *Proc. Natl. Acad. Sci. USA* 2003. **100**: 12307–12312.

20 **Shoemaker, J., Saraiva, M. and O'Garra, A.,** GATA-3 directly remodels the IL-10 locus independently of IL-4 in CD4+T cells. *J. Immunol.* 2006. **176**: 3470–3479.

21 **Rutz, S., Janke, M., Kassner, N., Hohnstein, T., Krueger, M. and Scheffold, A.,** Notch regulates IL-10 production by T helper 1 cells. *Proc. Natl. Acad. Sci. USA* 2008. **105**: 3497–3502.

22 **Akimzhanov, A. M., Yang, X. O. and Dong, C.,** Chromatin remodeling of interleukin-17 (IL-17)-IL-17F cytokine gene locus during inflammatory helper T cell differentiation. *J. Biol. Chem.* 2007. **282**: 5969–5972.

23 **Harrington, L. E., Hatton, R. D., Mangan, P. R., Turner, H., Murphy, T. L., Murphy, K. M. and Weaver, C. T.,** Interleukin 17-producing CD4+effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat. Immunol.* 2005. **6**: 1123–1132.

24 **Infante-Duarte, C., Horton, H. F., Byrne, M. C. and Kamradt, T.,** Microbial lipopeptides induce the production of IL-17 in Th cells. *J. Immunol.* 2000. **165**: 6107–6115.

25 **Annunziato, F., Cosmi, L., Santarlasci, V., Maggi, L., Liotta, F., Mazzinghi, B., Parente, E. *et al.*,** Phenotypic and functional features of human Th17 cells. *J. Exp. Med.* 2007. **204**: 1849–1861.

26 **Suryani, S. and Sutton, I.,** An interferon-gamma-producing Th1 subset is the major source of IL-17 in experimental autoimmune encephalitis. *J. Neuroimmunol.* 2007. **183**: 96–103.

27 **Manz, R., Assenmacher, M., Pfluger, E., Miltenyi, S. and Radbruch, A.,** Analysis and sorting of live cells according to secreted molecules, relocated to a cell-surface affinity matrix. *Proc. Natl. Acad. Sci. USA* 1995. **92**: 1921–1925.

28 **Assenmacher, M., Lohning, M. and Radbruch, A.,** Detection and isolation of cytokine secreting cells using the cytometric cytokine secretion assay. *Curr. Protoc. Immunol.*/edited by John E. Coligan *et al.* 2002. **Chapter 6**: Unit 6. 27.

29 **Rouvier, E., Luciani, M. F., Mattei, M. G., Denizot, F. and Golstein, P.,** CTLA-8, cloned from an activated T cell, bearing AU-rich messenger RNA instability sequences, and homologous to a herpesvirus saimiri gene. *J. Immunol.* 1993. **150**: 5445–5456.

30 **Hwang, E. S., Szabo, S. J., Schwartzberg, P. L. and Glimcher, L. H.,** T helper cell fate specified by kinase-mediated interaction of T-bet with GATA-3. *Science* 2005. **307**: 430–433.

31 **Szabo, S. J., Dighe, A. S., Gubler, U. and Murphy, K. M.,** Regulation of the interleukin (IL)-12R beta 2 subunit expression in developing T helper 1 (Th1) and Th2 cells. *J. Exp. Med.* 1997. **185**: 817–824.

32 **Ansel, K. M., Lee, D. U. and Rao, A.,** An epigenetic view of helper T cell differentiation. *Nat. Immunol.* 2003. **4**: 616–623.

33 **Gocke, A. R., Cravens, P. D., Ben, L. H., Hussain, R. Z., Northrop, S. C., Racke, M. K. and Lovett-Racke, A. E.,** T-bet regulates the fate of Th1 and Th17 lymphocytes in autoimmunity. *J. Immunol.* 2007. **178**: 1341–1348.

34 **Mathur, A. N., Chang, H. C., Zisoulis, D. G., Kapur, R., Belladonna, M. L., Kansas, G. S. and Kaplan, M. H.,** T-bet is a critical determinant in the instability of the IL-17-secreting T-helper phenotype. *Blood* 2006. **108**: 1595–1601.

35 **Grogan, J. L., Mohrs, M., Harmon, B., Lacy, D. A., Sedat, J. W. and Locksley, R. M.,** Early transcription and silencing of cytokine genes underlie polarization of T helper cell subsets. *Immunity* 2001. **14**: 205–215.

36 **Jones, B. and Chen, J.,** Inhibition of IFN-gamma transcription by site-specific methylation during T helper cell development. *EMBO J.* 2006. **25**: 2443–2452.

37 **Murayama, A., Sakura, K., Nakama, M., Yasuzawa-Tanaka, K., Fujita, E., Tateishi, Y., Wang, Y. *et al.*,** A specific CpG site demethylation in the human interleukin 2 gene promoter is an epigenetic memory. *EMBO J.* 2006. **25**: 1081–1092.

38 **Starnes, T., Robertson, M. J., Sledge, G., Kelich, S., Nakshatri, H., Broxmeyer, H. E. and Hromas, R.,** Cutting edge: IL-17F, a novel cytokine selectively expressed in activated T cells and monocytes, regulates angiogenesis and endothelial cell cytokine production. *J. Immunol.* 2001. **167**: 4137–4140.

39 **Park, H., Li, Z., Yang, X. O., Chang, S. H., Nurieva, R., Wang, Y. H., Wang, Y. *et al.*,** A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat. Immunol.* 2005. **6**: 1133–1141.

40 **Yang, X. O., Panopoulos, A. D., Nurieva, R., Chang, S. H., Wang, D., Watowich, S. S. and Dong, C.,** STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. *J. Biol. Chem.* 2007. **282**: 9358–9363.

41 **Richter, A., Lohning, M. and Radbruch, A.,** Instruction for cytokine expression in T helper lymphocytes in relation to proliferation and cell cycle progression. *J. Exp. Med.* 1999. **190**: 1439–1450.

Full correspondence: Dr. Hyun-Dong Chang, German Rheumatism Research Center, Charitéplatz 1, Berlin 10117, Germany
Fax: +49-30-28460-603
e-mail: chang@drfz.de

Additional correspondence: Prof. Dr. Andreas Radbruch, German Rheumatism Research Center, Charitéplatz 1, Berlin 10117, Germany
e-mail: radbruch@drfz.de

Supporting Information for this article is available at www.wiley-vch.de/contents.jc_2040/2008/38541_s.pdf

Received: 26/5/2008
Revised: 8/7/2008
Accepted: 22/7/2008

3.2 IFNγ und IL-12 kooperieren bei der Induktion von Th17+1-Zellen

Lexberg MH, Taubner A, Albrecht I, Lepenies I, Richter A, Kamradt T, Radbruch A*, **Chang HD***. 2010. IFN-γ and IL-12 synergize to convert *in vivo* generated Th17 into Th1/Th17 cells. *Eur J Immunol*. 40(11):3017-27.

In vivo und insbesondere bei chronischen Entzündungen wurden Zellen gefunden, die sowohl IL-17 als auch IFNγ exprimieren. Aufgrund der Koexpression von zwei als pro-inflammatorisch eingestuften Zytokinen und der Anhäufung bei chronischen Entzündungen wird diesen „Hybrid"-Zellen eine besonders pathogene Rolle zugeschrieben. Es war jedoch unklar wie diese Zellen entstehen. Wie wir bereits zeigen konnten kann in Th1-Zellen durch die Th17-instruierenden Signale TGF-β, IL-6 und IL-23 kein IL-17 induziert werden. *In vitro* differenzierte Th17-Zellen werden aber in Gegenwart von IL-12 zu Th1 Zellen „umprogrammiert", mit Induktion der T-bet- und IFNγ-Expression und dem kompletten Verlust der Expression von RORγt und IL-17, während *in vivo* differenzierte Th17-Zellen stabil und refraktär gegenüber IL-12 bleiben.
Wir haben die Unterschiede zwischen *in vitro* und *in vivo* generierten Th17-Zellen genauer untersucht und konnten zeigen, dass *in vivo* generierte Th17-Zellen die IL-12Rβ2-Kette nicht exprimieren und daher nicht auf IL-12 reagieren können. Sie exprimieren aber den Rezeptor für IFNγ. Stimulation der Th17-Zellen mit IFNγ führte zu einer Induktion der IL12Rβ2-Expression und stellte die Reaktivität zu IL-12 wieder her. Eine anschließende Stimulation der mit IFNγ vorbehandelten Th17-Zellen mit IL-12 führte zur Induktion der T-bet- und IFNγ-Expression. Interessanterweise behielten die mit IL-12-stimulierten Th-Zellen die Expression von RORγt und IL-17, d.h. sie wurden zu hybriden Th17+1 Zellen, die RORγt und T-bet koexprimieren. Somit konnten wir einen Signalweg identifizieren, nämlich die Aktivierung von Th17-Zellen in Gegenwart von IFNγ und IL-12, der zur Differenzierung von Th17+1-Zellen führt.

European Journal of Immunology

IFN-γ and IL-12 synergize to convert *in vivo* generated Th17 into Th1/Th17 cells

Maria H. Lexberg[1], Annegret Taubner[2], Inka Albrecht[1], Inga Lepenies[1], Anne Richter[3], Thomas Kamradt[2], Andreas Radbruch[1] and Hyun-Dong Chang*[1]*

[1] Deutsches Rheuma-Forschungszentrum Berlin, A Leibniz Institute, Berlin, Germany
[2] Institute of Immunology, Friedrich Schiller University, Jena Medical School, Jena, Germany
[3] Miltenyi Biotec GmbH, Bergisch Gladbach, Germany

Th1 and Th17 cells are distinct lineages of effector/memory cells, imprinted for re-expression of IFN-γ and IL-17, by upregulated expression of T-bet and retinoic acid-related orphan receptor γt (RORγt) , respectively. Apparently, Th1 and Th17 cells share tasks in the control of inflammatory immune responses. Th cells coexpressing IFN-γ and IL-17 have been observed *in vivo*, but it remained elusive, how these cells had been generated and whether they represent a distinct lineage of Th differentiation. It has been shown that *ex vivo* isolated Th1 and Th17 cells are not interconvertable by TGF-β/IL-6 and IL-12, respectively. Here, we show that *ex vivo* isolated Th17 cells can be converted into Th1/Th17 cells by combined IFN-γ and IL-12 signaling. IFN-γ is required to upregulate expression of the IL-12Rβ2 chain, and IL-12 for Th1 polarization. These Th1/Th17 cells stably coexpress RORγt and T-bet at the single-cell level. Our results suggest a molecular pathway for the generation of Th1/Th17 cells *in vivo*, which combine the pro-inflammatory potential of Th1 and Th17 cells.

Key words: Cytokine memory · IL-17 · T-cell differentiation · Th1/Th17 cells

Supporting Information available online

Introduction

Th1 cells, with a memory for IFN-γ expression and determined by the master transcription factor T-bet, are considered to be essential for protection against intracellular pathogens, and had been viewed as the major pathogenic drivers of chronic autoimmune inflammation, *e.g.* EAE [1–3], uveitis [4] or colitis [5]. Recently, Th17 cells, with a memory for expression of IL-17 and determined by the transcription factor retinoic acid related orphan receptor γt (RORγt), have been identified as another pathogenic Th-cell lineage driving pathogenesis in these autoimmune models [6, 7]. Th17 cells contribute to inflammation through the recruitment of neutrophils and the induction of secretion of pro-inflammatory mediators such as IL-6, IL-8, TNF-α, IL-1β, CXCL1, CXCL10 and matrix metalloproteinases from tissue cells (reviewed in [8]). Th1 cells contribute to inflammation by activation of macrophages [9]. The concerted action of IFN-γ and IL-17 has been shown to be essential in the effective induction and maintenance of autoimmunity [10, 11], *e.g.* Th1 cells being required for the recruitment of Th17 cells into the central nervous system in EAE.

In inflamed tissue of autoimmune patients, Th cells co-expressing IFN-γ and IL-17 have been identified [12–14].

Correspondence: Dr. Hyun-Dong Chang
e-mail: chang@drfz.de

*These authors have contributed equally to this study.

However, the genesis and stability of such Th cells had remained enigmatic. *In vitro*, the differentiation of naïve Th cells into Th1 or Th17 cells is mutually exclusive, using the polarizing signals identified so far. TGF-β blocks the induction of expression of T-bet and IFN-γ [15]. IFN-γ blocks the differentiation of naïve Th cells into Th17 cells [16]. We have previously shown that murine Th17 cells isolated *ex vivo* cannot be converted into Th1 cells by IL-12, or into Th2 cells by IL-4 [17], supporting the notion that Th17 cells are a distinct, stable and independent lineage of Th memory/effector differentiation. It has, however, been demonstrated that human Th-cell clones coexpressing IL-17 and IL-4 can be generated by stimulation of Th17 containing $CCR6^+CD161^+$ Th cells with IL-4 [18].

Here, we demonstrate that Th17 cells can be induced to develop further, into Th1/Th17 cells, by the combined action of IFN-γ and IL-12. We show that *ex vivo* isolated Th17 cells lack IL-12Rβ2 expression and are not responsive to IL-12 alone. However, the expression of IL-12Rβ2 can be induced with IFN-γ, restoring IL-12 responsiveness. Stimulation of *ex vivo* isolated Th17 cells with IFN-γ and IL-12 results in stable induction of T-bet and functional imprinting of the *Ifnγ* gene for re-expression. Expression of RORγt is maintained, as well as functional imprinting of the *Il17* gene for re-expression. Individual Th1/Th17 cells stably coexpress T-bet and RORγt. Our results suggest a molecular pathway for the generation of Th1/Th17 cells *in vivo*, and define Th1/Th17 cells as a functionally distinct Th population, combining the pro-inflammatory potential of Th1 and Th17 cells.

Results

In vivo, Th17 cells do not express IL-12Rβ2 and do not respond to IL-12

Although IL-17-expressing cells isolated from cultures stimulated *in vitro* respond to subsequent stimulation with IL-12 with gain of IFN-γ expression and loss of IL-17 expression (Fig. 1A, upper panel), IL-17 expressing cells directly isolated *ex vivo* maintained IL-17 expression and could not be induced to express IFN-γ in the presence of IL-12 (Fig. 1A, lower panel) [17, 19]. To identify the molecular mechanism of refraction of *in vivo* generated Th17 cells to conversion by IL-12, we here compared the expression of genes relevant for IL-12 signaling by Th17 cells generated *in vitro*, and $CD4^+$ T cells isolated directly *ex vivo* according to secretion of IL-17, using the cytometric cytokine secretion assay for IL-17, as described earlier [17]. Expression levels of the lineage determining transcription factors for Th1 and Th17 cells, T-bet and RORγt, respectively, the IFN-γ receptor 2 (IFN-γR2) and the inducible IL-12Rβ2 chain were determined by quantitative PCR (Fig. 1B). Expression of the transcription factor RORγt in *ex vivo* isolated $IL\text{-}17^+$ T cells was threefold higher as compared with *in vitro*-generated $IL\text{-}17^+$ Th cells. Increased T-bet mRNA levels (threefold) were detected in the *ex vivo*-isolated Th17 cells compared with *in vitro*-generated Th17 cells (Fig. 1B). Directly, *ex vivo* isolated $IL\text{-}17^+$ Th cells expressed fivefold less IL-12Rβ2 transcripts than *in vitro*-generated Th17 cells (Fig. 1B). Allowing the cells to rest did not significantly increase IL-12Rβ2 expression of *ex vivo* isolated Th17 cells (data not shown), suggesting that *Il12rβ2* chain expression of Th17 cells is downregulated constitutively *in vivo*. *Ex vivo* isolated $IL\text{-}17^+$ Th cells stimulated with IL-12 did not respond by STAT4 phosphorylation, demonstrating the absence of a functional IL-12 receptor on these cells (Fig. 1C). On the contrary, stimulation of *ex vivo* isolated IFN-γ^+ Th cells resulted in STAT4 phosphorylation in more than 50% of the cells (Supporting Information Fig. 2). *In vitro*-generated Th17 cells responded to IL-12, by phosphorylation of STAT4 in all cells (Fig. 1C), and expression of IFN-γ by more than 40% of the cells ([17] and data not shown).

To confirm that downregulation of IL-12Rβ2 chain expression is not a transient consequence of TCR activation [20, 21], we directly isolated splenic Th cells based on the surface expression of IL-12Rβ2 (Fig. 2A, gating for viable $CD4^+$ Th cells shown in Supporting Information Fig. 1D) and then quantified cells expressing intracellular IL-17 upon reactivation, among the IL-12Rβ2^+ and IL-12β2^- Th cells. Within the IL-12Rβ2^-CD4^+ T cells, we could detect 0.66% IL-17-expressing cells, whereas 0.18% of the IL-12Rβ2^+ Th cells expressed IL-17 (Fig. 2B). In terms of absolute cell numbers, we could detect $4 \times 10^4 \pm 6 \times 10^3$ IL-17-expressing cells *per* murine spleen, which did not coexpress IL-12Rβ2, and $2 \times 10^3 \pm 1 \times 10^3$ Th cells coexpressing IL-17 and IL-12Rβ2 (Fig. 2C). This means that *in vivo* 95% of murine Th17 cells found in the spleen do not express a functional IL-12 receptor.

IFN-γ induces IL-12Rβ2 expression by Th17 cells and restores responsiveness to IL-12

The *Ifnγr2* gene was three- to four-fold higher expressed by Th17 cells, both *in vivo* and *in vitro* generated, than in Th1 cells (data not shown). This is in accordance with the previous reports demonstrating upregulation of *Ifnγr* expression by IL-6 [22] and downregulation of *Ifnγr* expression in Th1 cells *in vitro* [23]. In *in vitro*-generated and directly *ex vivo* isolated Th17 cells, the IFN-γ receptor is functional. STAT1 phosphorylation was induced upon stimulation with IFN-γ (Fig. 1C), whereas Th1 cells did not respond to IFN-γ (data not shown). Interestingly, IFN-γ-induced activation of STAT1 was not sufficient to induce significant expression of *Ifnγ* (Fig. 3A), unlike shown previously in naïve Th cells [20, 24]. Stimulation with IFN-γ did not change the expression level of RORγt but resulted in a detectable increase of T-bet expression as assessed by intracellular immunofluorescence (Fig. 3B). However, in *ex vivo* solated Th17 cells, IFN-γ induced the expression of *Il12Rβ2* (Fig. 3C) and functionally restored responsiveness to IL-12 (Fig. 3D) as has been shown for Th1 and Th2 cells before [25]. When prestimulated with IFN-γ, IL-12 induced phosphorylation of STAT4 in more than 50% of the cells (Fig. 3D). IL-12, as previously shown [17], did not lead to significant induction of IFN-γ expression (Fig. 3A), nor upregulation of T-bet expression or changes in RORγt expression (Fig. 3B), upregulation of *Il12Rβ2* (Fig. 3C) or responsiveness to IL-12 (Fig. 3D).

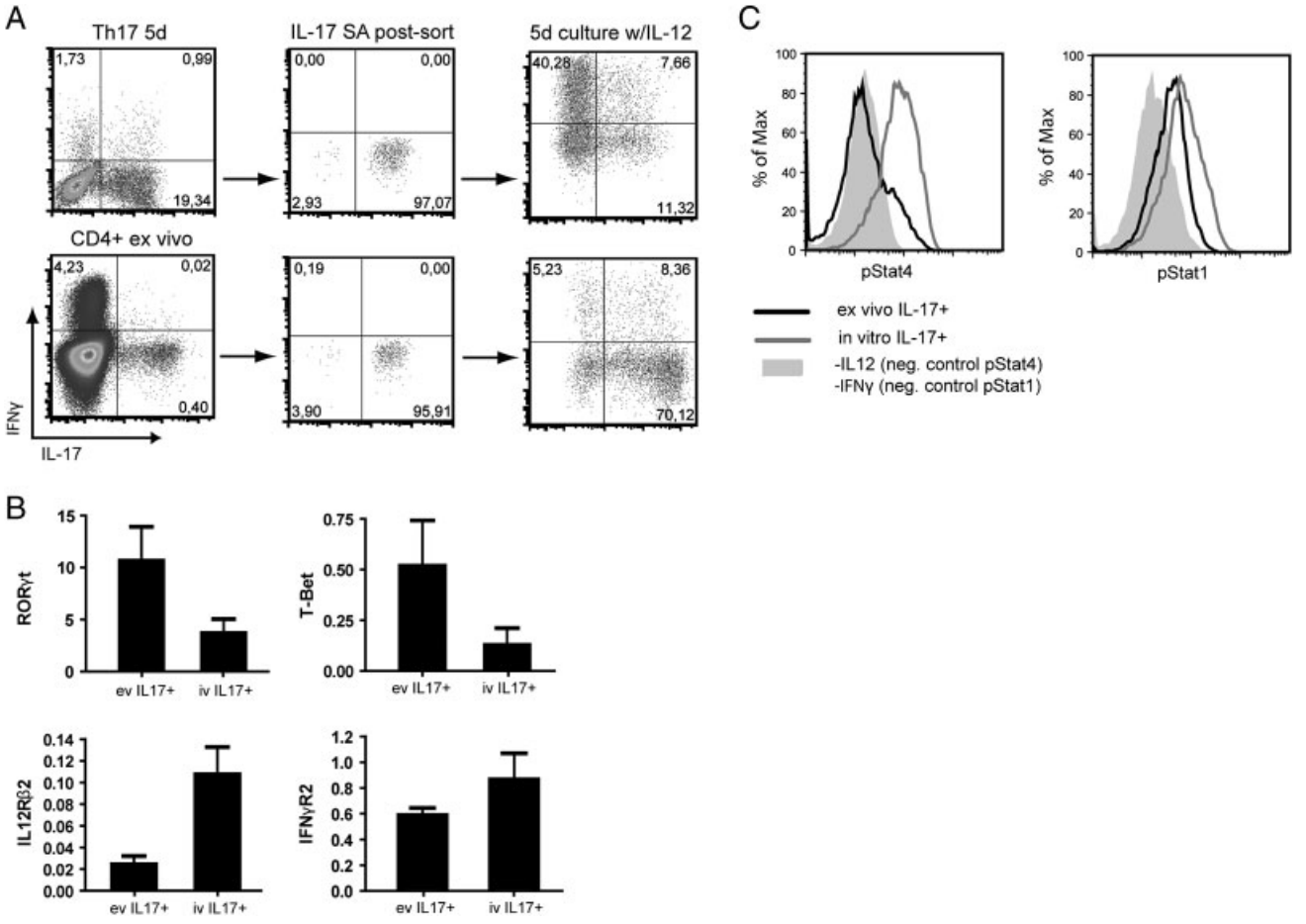


Figure 1. *Ex vivo* isolated Th17 cells express a functional IFN-γ receptor but lack a functional IL-12 receptor. (A) $CD4^+$ T cells from spleen and lymph nodes from ex-breeder BALB/c mice and Th17 cells, generated *in vitro* by stimulating naïve $CD4^+CD62L^{high}$ cells with TGF-β, IL-6, IL-23, anti-IL-4 and anti-IFN-γ for 5 days, were stimulated for 5 h with PMA/ionomycin and stained intracellularly for IL-17 and IFN-γ (gated on $CD4^+$ lymphocytes, Supporting Information Fig. 1A and B). IL-17-producing Th cells were isolated with an IL-17 secretion assay, stimulated with anti-CD3/anti-CD28 and cultured in the presence of IL-12 and absence of IL-4 and IFN-γ. Data are representative of five independent experiments. (B) mRNA expression of RORγt, IL-12Rβ2, IFN-γR2 and T-bet was determined in IL-17-secreting cells isolated directly *ex vivo* (ev) and from *in vitro* (iv)-induced Th17 cells and normalized to the housekeeping gene hypoxanthine guanine phosphoribosyl transferase. Data show mean ± SD of three independent experiments. (C) IL-17-secreting cells isolated *ex vivo* and generated *in vitro* were rested for 2 days in the absence of IL-4, IFN-γ and IL-12. The cells were then stimulated for 30 min with IL-12 prior to intracellular staining of pSTAT4 or 15 min with IFN-γ for staining of pSTAT1. Scatter characteristics are shown in Supporting Information Fig. 1C. Cells incubated with culture medium alone served as negative control. Data are representative of three independent experiments.

IFN-γ and IL-12 synergize to induce Th1/Th17 cells

Following restoration of responsiveness to IL-12 by prestimulation with IFN-γ, expression of the *Ifnγ* gene could be induced by IL-12 stimulation (Fig. 4A). All of the cells uniformly had upregulated T-bet expression, as determined by intracellular immunofluorescence, and 50% expressed intracellular IFN-γ. Although inducing expression of T-bet and IFN-γ in *ex vivo* isolated Th17 cells, combined IFN-γ and IL-12 signaling did not suppress expression of RORγt. All cells uniformly continued to express RORγt (Fig. 4B and C). Upon restimulation, they also re-expressed *Il17*. The frequencies of IL-17-producing cells dropped from 75% (±10%) to 38% (±6%). In total, 20% of the cells expressed only IL-17, 20% IL-17 and IFN-γ, and 30% only IFN-γ (Fig. 4A). The frequency of cells coexpressing IL-17 and IFN-γ observed (obs: 20%) corresponds to the frequency one would expect from the random coincidence of coexpression (exp: 20%), suggesting that the expression of IL-17 and IFN-γ is neither positively nor negatively correlated [26]. Repeated stimulation of *ex vivo* isolated Th17 cells from IFN-γR2-deficient mice with IL-12 alone did not lead to a significant induction of IFN-γ in these cells (Supporting Information Fig. 3). We also exclude the selective outgrowth of pre-existing IFN-γ expressing cells as stimulation with IL-12 of isolated IL-17-expressing cells depleted of $IFN\text{-}\gamma^+$ cells also resulted in the induction of cells coexpressing IFN-γ and IL-17 (Supporting Information Fig. 4).

Th1/Th17 cells coexpress T-bet and RORγt

To analyze the stability of Th1/Th17 cells generated *in vivo*, we have immunized wild-type mice with peptide derived from

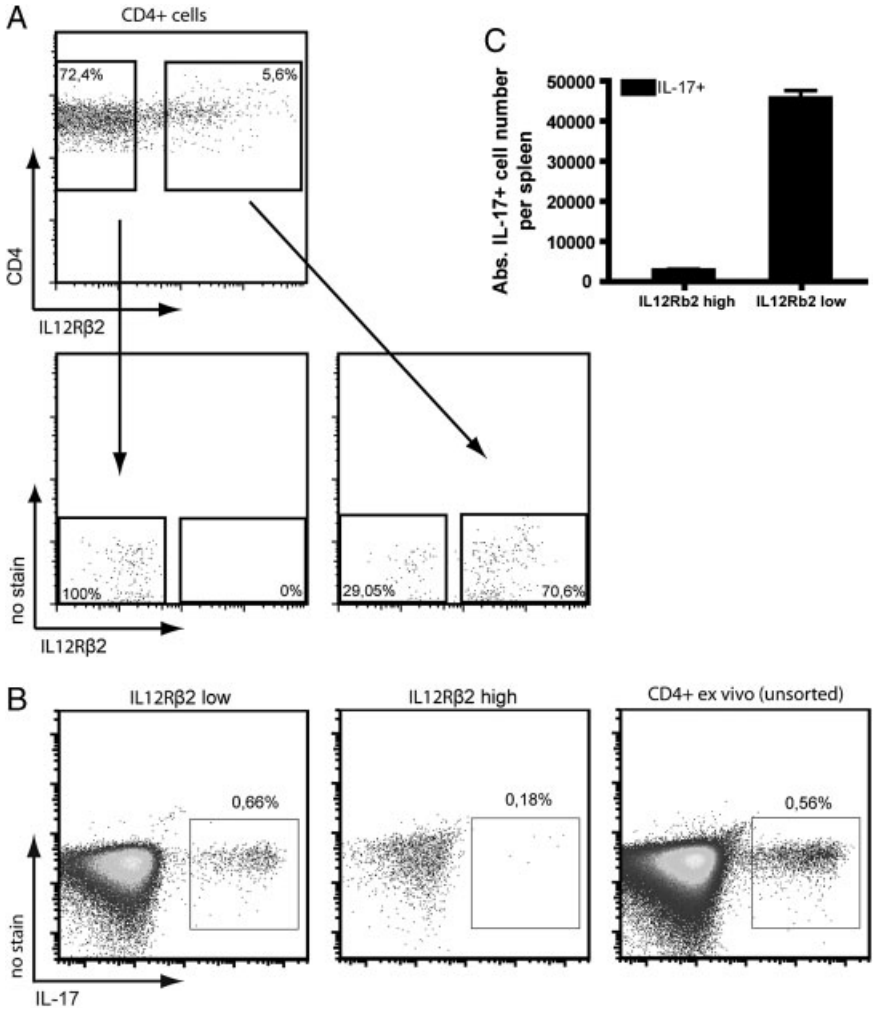


Figure 2. IL-17-expressing Th cells are enriched within the IL-12Rβ2^{low} subset. (A) CD4$^+$ T cells from spleen and lymph nodes from ex-breeder BALB/c mice were isolated with CD4 MACS beads. The cells were stained for IL-12Rβ2 and IL-12Rβ2^{high} and IL-12Rβ2^{low} CD4$^+$ Th cells were isolated by FACS sorting. Data are representative of three independent experiments. (B) The sorted IL-12Rβ2^{high} and IL-12Rβ2^{low} CD4$^+$ Th cells were stimulated with PMA/ionomycin for 4 h for recall expression of cytokines. The percentage of IL-17-producing cells *per* spleen was determined by intracellular cytokine staining. (C) Absolute cell numbers of IL-17-producing cells were determined with a MACSQuant Analyzer. Data show mean ± SD of three independent experiments.

myelin oligodendrocyte glycoprotein$_{35–55}$ in complete Freund's adjuvant. Before onset of any clinical symptoms of EAE (Fig. 5A), we have assessed the expression of the cytokines IFN-γ and IL-17 in splenic CD4$^+$ Th cells at day 7 post-immunization. Following restimulation with PMA and ionomycin, 5.56% of the splenic CD4$^+$ Th cells expressed only IFN-γ, whereas 2.58% expressed only IL-17. In total, 0.62% of all splenic CD4$^+$ Th cells coexpressed IL-17 and IFN-γ (Fig. 5A). On the contrary, in age-matched, unimmunized control mice, no IL-17-expressing or IFN-γ/IL-17 coexpressing CD4$^+$ Th cells could be detected (Fig. 5A). Using an IFN-γ/IL-17 double secretion assay, we have isolated from spleen Th cells expressing either IFN-γ only, IL-17 only or coexpressing IL-17 and IFN-γ (Fig. 5B). Th cells expressing only IFN-γ uniformly expressed elevated T-bet levels (Δgeo mean of 1152 compared with negative control), whereas RORγt expression was low but detectable. IL-17 only-expressing Th cells expressed high RORγt levels (Δgeo mean of 3387) and detectable levels of T-bet (Δgeo mean of 518), similar to the *ex vivo* isolated Th17 cells shown in Fig. 1. Th cells expressing both IL-17 and IFN-γ uniformly expressed elevated levels of RORγt and T-bet (Δgeo mean of 2368 and 1138, respectively) (Fig. 5C). To confirm the intracellular staining, the mRNA expression of RORγt and T-bet in the three isolated subsets was quantified. T-bet and RORγt expression in the IL-17$^+$IFN-γ$^+$ Th1/Th17 cells was at similar levels as T-bet and RORγt levels in IFN-γ single-positive and IL-17 single-positive Th cells, respectively (Fig. 5D). Analysis of the expression levels of RORα, IL-12Rβ2, IFN-γR2 and L-23R also confirmed that Th1/Th17 cells share the properties of IL-17$^+$ and IFN-γ$^+$ Th cells (Supporting Information Fig. 5). The directly *ex vivo* isolated Th1/Th17 cells were then cultured without the addition of any blocking antibodies and exogenous cytokines (neutral) for 5 or 10 days. Upon restimulation with PMA/ionomycin, 23 ± 1% of the cells coexpressed IL-17 and IFN-γ, 55 ± 4% only IFN-γ and 12 ± 3% only IL-17 after 5 days (Fig. 5E).

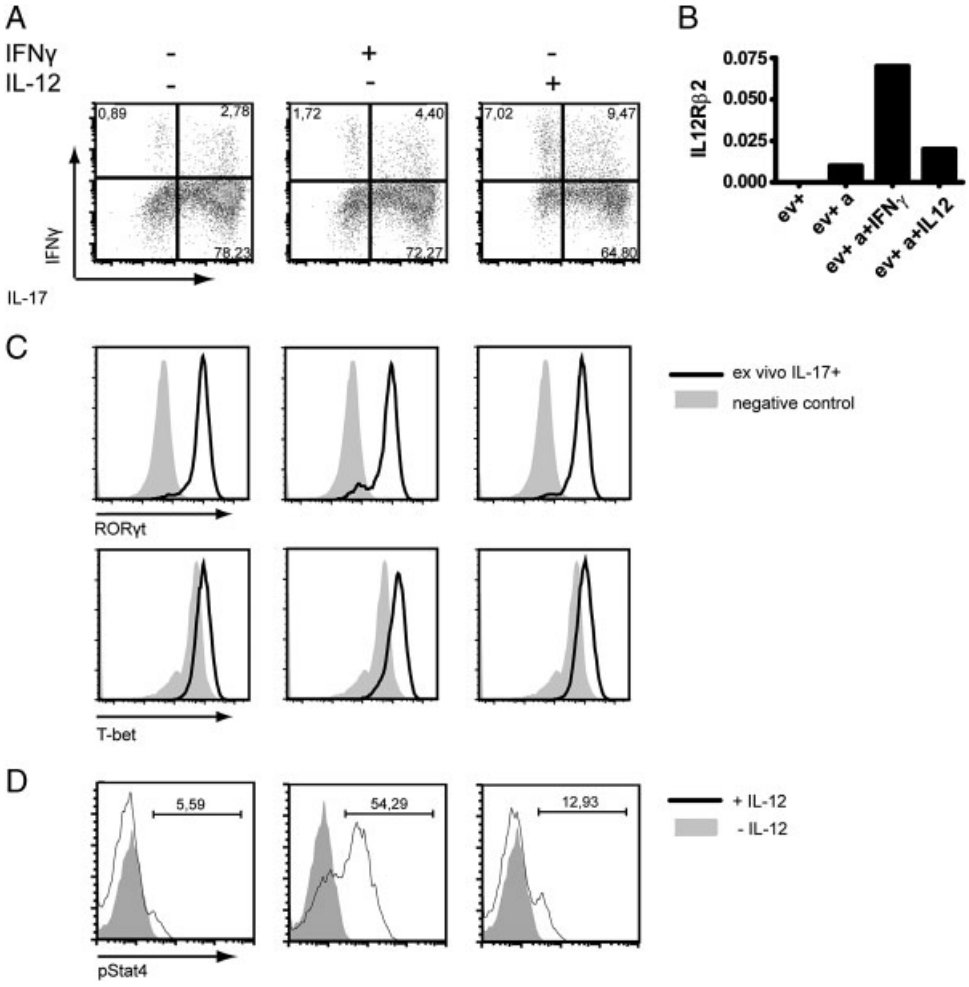


Figure 3. IL-12Rβ2 expression is induced in *ex vivo* isolated Th17 cells by IFN-γ. (A) *Ex vivo* isolated IL-17$^+$ cells were cultured in the absence of IFN-γ and IL-12 or in the presence of IFN-γ or IL-12 only for 5 days and stained intracellularly for IFN-γ and IL-17 expression. IL-4 was blocked under all conditions. Data are representative of five independent experiments. (B) mRNA was isolated from directly isolated IL-17$^+$ Th cells, IL-17$^+$ Th cells cultured in the absence of cytokines, in the presence of IFN-γ only or IL-12 only. IL-12Rβ2 expression was determined by real-time PCR. Experiment is representative of two independent experiments. (C) Intracellular RORγt and T-bet expression was measured after 5 days cell culture under the indicated conditions. Data are representative of three independent experiments. (D) pSTAT4 in response to IL-12 was measured by intracellular staining in IL-17$^+$ Th cells cultured under the indicated conditions. Data are representative of three independent experiments.

The relative distribution of cytokine producers was maintained at similar levels after day 10 (12±6% coexpressing IL-17 and IFN-γ, 59±14% only IFN-γ and 14±6% only IL-17) (Fig. 5F). T-bet expression was maintained uniformly over the 10 days of culture. RORγt expression was also maintained, although to a lesser degree in some of the cells, most of which corresponded to the IFN-γ-only cells (data not shown).

We have also directly isolated Th1/Th17 cells *ex vivo* from human blood, and analyzed the stability of RORγt and T-bet expression, as well as IL-17 and IFN-γ re-expression *in vitro*. Th1/Th17 cells were isolated according to coexpression of IFN-γ and IL-17, using the IFN-γ/IL-17 double secretion assay (Fig. 6A). Viable Th1/Th17 cells were enriched from 1% of CD4$^+$ cells to 94% purity, and these cells expressed both RORγt and T-bet (Fig. 6A and B). Th cells expressing neither cytokine expressed neither RORγt nor T-bet. IFN-γ-only positive cells expressed only T-bet and IL-17-only positive T cells expressed only RORγt (Supporting Information Fig. 6). Directly *ex vivo* isolated Th1/Th17 cells were then cultured under neutral conditions for 5 or 10 days. Upon restimulation with PMA/ionomycin, about 40% of the cells coexpressed IL-17 and IFN-γ, 26% only IFN-γ and 10% only IL-17 (Fig. 6C). Again, more than 50% of the cells uniformly maintained coexpression of T-bet and RORγt, the canonical Th1 and Th17 master transcription factors over the 10 days of *in vitro* culture (Fig. 6D).

Discussion

We here provide a molecular mechanism for the generation of a distinct Th-cell population characterized by the additive phenotypes of Th1 and Th17 cells, the Th1/Th17 cells. Th1/Th17 cells are characterized by the coexpression of the cytokines IFN-γ and IL-17 and the lineage-defining and -determining transcription

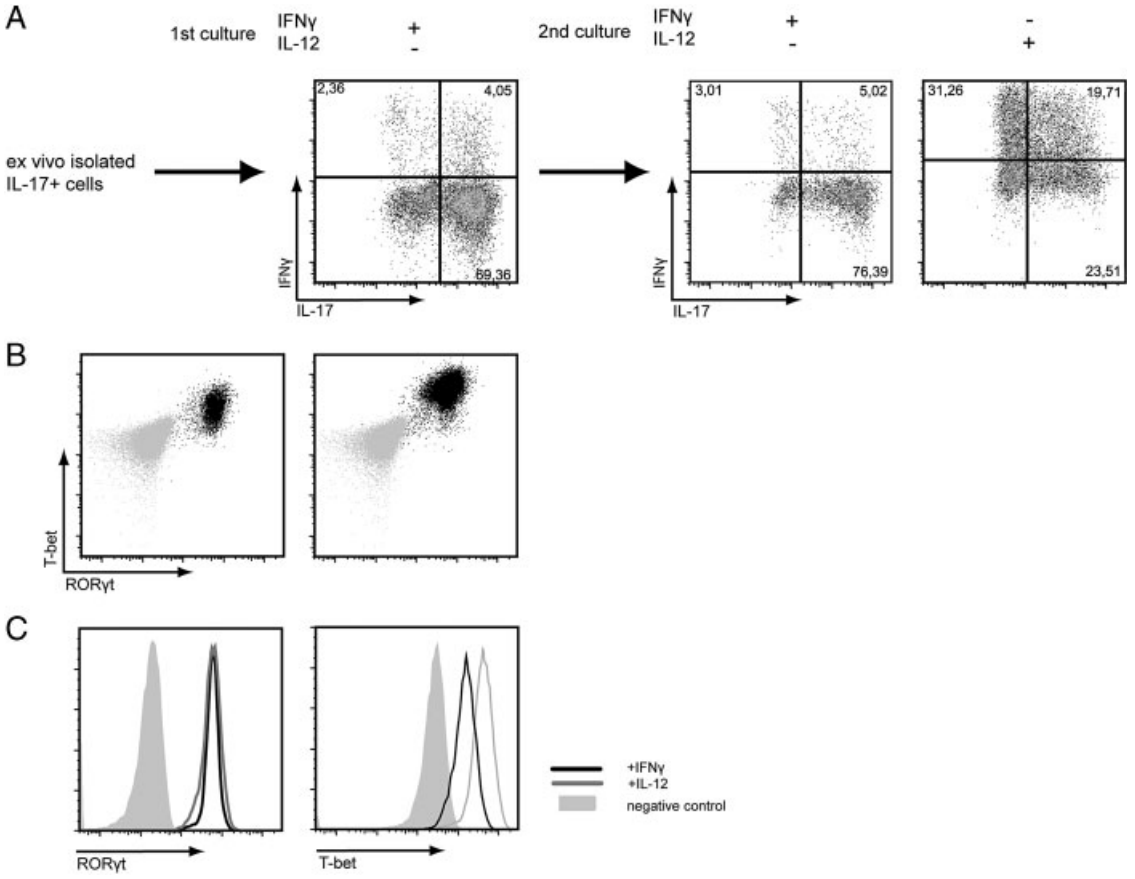


Figure 4. IFN-γ and IL-12 synergize to induce Th1/Th17 cells. (A) Directly *ex vivo* isolated IL-17$^+$ CD4 T cells were cultured for 5 days in the presence of IFN-γ and then restimulated in the presence of either IFN-γ or IL-12 for another 5 days. Shown is the intracellular staining for IFN-γ and IL-17 expression after each culture. Data are representative of four independent experiments. (B and C) RORγt and T-bet were stained intracellularly following the second culture. Data are representative of four independent experiments.

factors T-bet and RORγt. Th1/Th17 cells develop from Th17 cells upon synergistic action of IFN-γ, required for the upregulation of the IL-12Rβ2 chain and IL-12. IL-12Rβ2 signaling is required for stable imprinting of Th1 cells [20].

The differentiation and maintenance of Th lineage phenotypes, in particular their memory for cytokine expression has been intensely studied [26, 27]. Several mechanisms have been identified, such as the inhibition of the Th2 master transcription factor GATA-3 through T-bet-mediated phosphorylation [28], the GATA-3-mediated downregulation of the IL-12Rβ2 chain [29] or STAT4 [30], or the sequestering of cytokine genes into heterochromatin [31], which restrict the plasticity of differentiated Th lineage cells. This has led to the overall picture that once fully differentiated cells of the various Th lineages are committed and resistant to conversion into other lineages [32]. We have shown previously that Th17 cells which have been generated *in vivo* are refractory to Th1 and Th2 polarization *ex vivo* [17], whereas *in vitro*-generated murine Th17 cells and human Th17 cells show a great degree of plasticity [19, 33]. To clarify this apparent discrepancy, here we demonstrate that *in vivo* generated Th17 cells have downregulated the IL-12Rβ2 chain and do not respond to IL-12. However, they still express the IFN-γ receptor and respond to IFN-γ signaling. IFN-γ signaling induces the upregulation of expression of T-bet and of the IL-12Rβ2 chain [20, 24, 34]. In this regard, *in vivo* generated Th17 cells behave like naïve Th cells [20]. Naïve Th cells were shown to require sequential activation with IFN-γ and IL-12, resulting in two waves of T-bet induction, whereby IL-12-induced T-bet is required for induction of IFN-γ expression and imprinting of the *Ifnγ* gene for re-expression [20]. Unlike naïve Th cells, *in vivo* generated Th17 cells maintain their enhanced expression of RORγt and expression of IL-17.

It remains to be shown how expression of the IL-12Rβ2 chain is downregulated in Th17 cells *in vivo*. Evidence has been provided that IL-17 itself directly [35], or indirectly, by inducing APC to release as yet unidentified factors [36], downregulates IL-12Rβ2 chain expression in activated Th cells. For *in vitro*-generated and -maintained Th17 cells, we could not confirm these observations. Neither was expression of the IL-12Rβ2 chain downregulated on such cells, nor did blocking of IL-17 upregulate its expression (data not shown).

The downregulation of the IL-12Rβ2 chain also apparently precludes Th1 polarization of Th2 cells. It has been shown for murine and human Th2 cells that IFN-γ and IL-12 in synergy can convert them into Th2/Th1 cells [25, 37, 38], enabling the immune

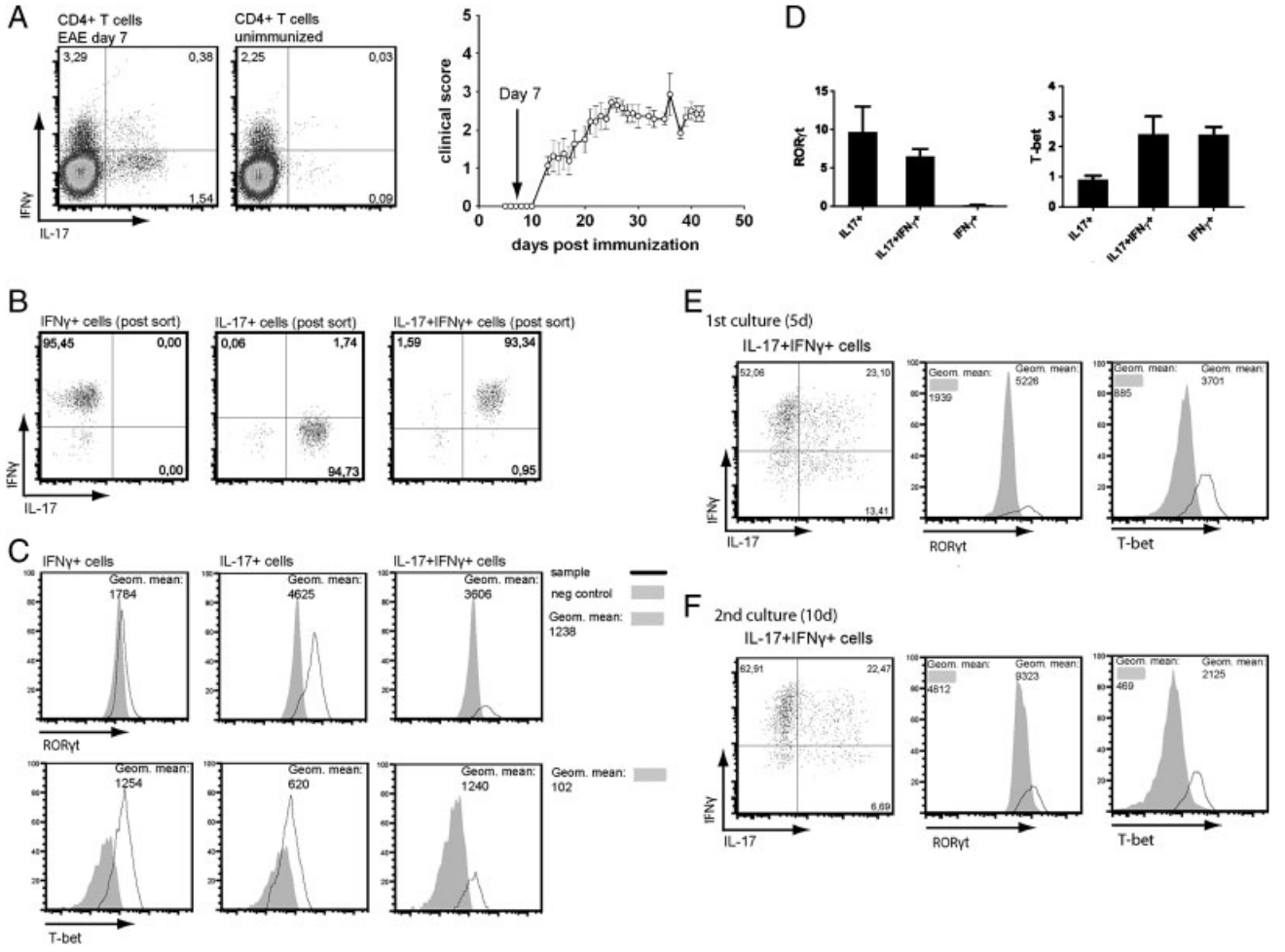


Figure 5. *Ex vivo* isolated murine Th1/Th17 cells coexpress RORγt and T-bet. (A) EAE was induced in C57BL/6 mice and cytokine expression on day 7 after immunization was measured in $CD4^+$ T cells from spleen and lymph nodes after stimulating the cells with PMA/Iono for 4 h. The cells were analyzed before any clinical symptoms of disease were measurable. (B) IFN-γ^+IL-17^-, IL-17^+IFN-γ^- and IL-17^+IFN-γ^+ $CD4^+$ T cells were isolated combining the IL-17 and IFN-γ secretion assay. (C) RORγt and T-bet were stained intracellularly in the different subsets. (D) mRNA was isolated from *ex vivo* isolated IL-17^+, IL-17^+IFN-γ^+ and IFN-γ^+ Th cells. Relative expression of RORγt and T-bet was determined by real-time PCR. Data show mean ± SD of three independent experiments. (E) IL-17^+IFN-γ^+ $CD4^+$ Th cells were stimulated with anti-CD3/anti-CD28/APC and cultured under neutral conditions (anti-IL-4, anti-IFN-γ and anti-IL-12) for 5 days. The cells were restimulated with PMA/Iono for 4 h and cytokine, T-bet and RORγt expression was measured. (F) The cells from (E) were restimulated with anti-CD3/anti-CD28/APC and cultured under neutral conditions (anti-IL-4, anti-IFN-γ and anti-IL-12) for another 5 days. Cytokine, T-bet and RORγt expression was measured after restimulation with PMA/Iono for 4 h. Data are representative of five independent experiments.

system to combine properties of both Th lineages in individual cells in immune responses to viruses [37] or intracellular bacteria [39]. Suryani and Sutton have suggested that IL-17/IFN-γ coexpressing cells are derived from Th1 cells, gaining the ability to express IL-17 [40]. Although we do not exclude this option here, the conversion of Th1 cells into Th1/Th17 cells must require signals different from the canonical Th17 differentiation signals TGF-β and IL-6. Neither *in vitro*-generated Th1 cells [17], nor *ex vivo* isolated Th1 cells, as we show here (Supporting Information Fig. 7), can be induced to express IL-17, by combined action of TGF-β, IL-6 and IL-23. In addition, upon adoptive transfer, Th1 cells are not converted into Th1/Th17 cells *in vivo* [41].

The physiological advantage of Th1/Th17 cells over Th1 and Th17 cells could be their combined effector repertoire on the single-cell level, coexpressing IFN-γ and IL-17, but also chemokine receptors of both Th1 and Th17 cells [41, 42], *i.e.* CCR2, CCR5 and CXCR3 of Th1 and CCR4 and CCR6 of Th17 cells, allowing them to deliver their cytokines at noncanonical locations. For example, Th1/Th17 cells could deliver IL-17 into inflamed tissue, attracted by the CXCR3 ligands CXCL9, 10 or 11 [43]. Indeed, it has been suggested in recent publications that Th17 cells expressing IFN-γ have pro-inflammatory properties clearly distinct from Th1 or Th17 cells. Th1/Th17 cells could more efficiently induce the expression of inflammatory mediators on epithelial cells [44] and were found to preferentially accumulate in the CNS of MS–infected patients and EAE mice compared with IFN-γ or IL-17 single producers [45].

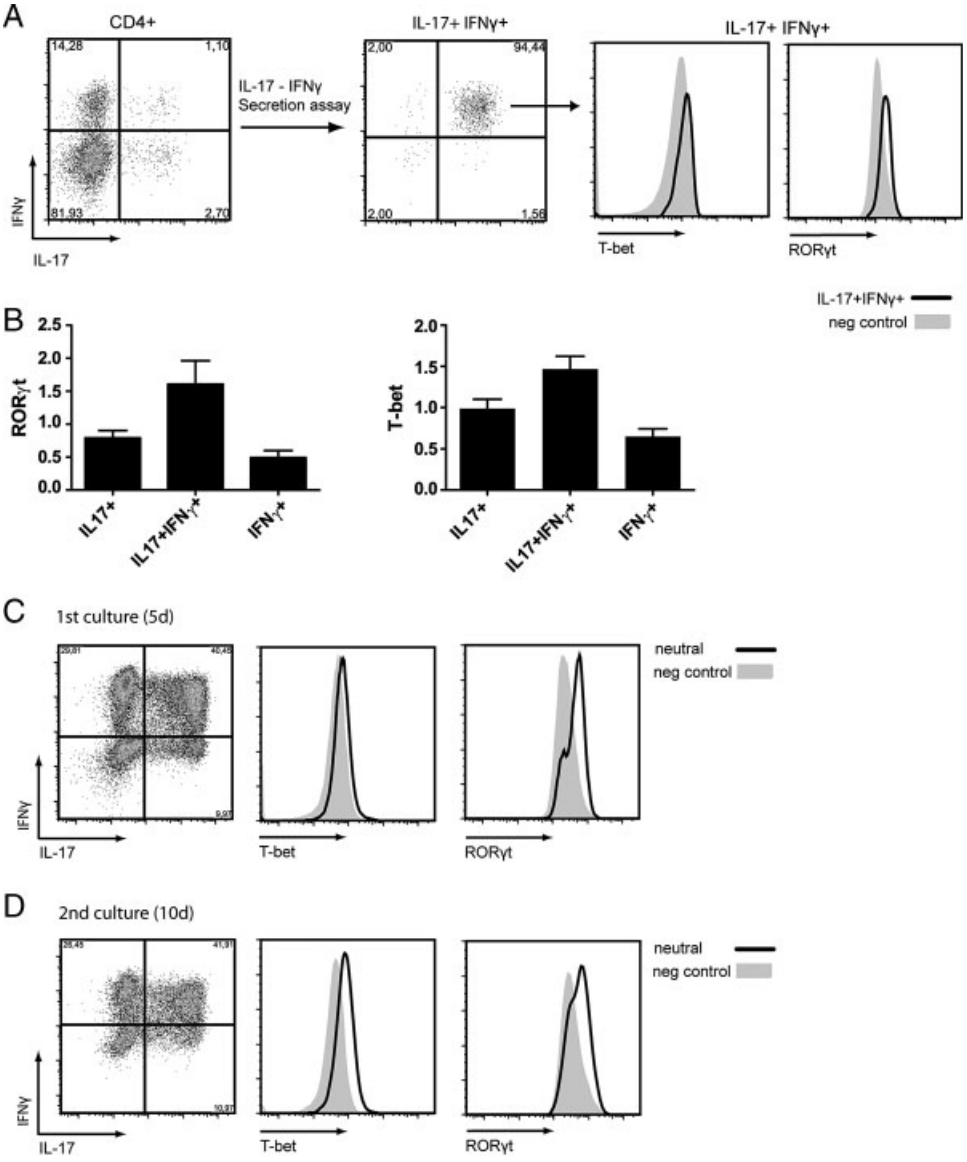


Figure 6. *Ex vivo* isolated human Th1/Th17 cells coexpress RORγt and T-bet. (A) Human CD4$^+$ T cells coexpressing IL-17 and IFN-γ were isolated from peripheral blood using the IL-17 and IFN-γ secretion assays and expression of RORγt and T-bet was measured. Data are representative of five independent experiments. (B) mRNA was isolated from *ex vivo* isolated IL-17$^+$, IL-17$^+$IFN-γ$^+$ and IFN-γ$^+$ human Th cells. Relative expression of RORγt and T-bet was determined by real-time PCR. Data show mean ± SD of four independent experiments. (C) IL-17$^+$IFN-γ$^+$ CD4$^+$ Th cells were cultured under neutral conditions (anti-IL-4 and anti-IFN-γ) for 5 days. The cells were restimulated with PMA/Iono for 6 h and cytokine, T-bet and RORγt expression was measured. (D) The cells from (B) were stimulated with anti-CD3/anti-CD28 and cultured under neutral condition (anti-IL-4 and anti-IFN-γ) for another 5 days. IL-17, IFN-γ, RORγt and T-bet expression was assessed by intracellular staining. Data are representative of five independent experiments. Gating strategy is shown in Supporting Information Fig. 1E.

Materials and methods

Mice and murine antibodies

Specific-pathogen-free 6- to 8-wk old, ex-breeder BALB/c and C57BL/6J mice were obtained from Charles River (Sulzburg, Germany). Mice were sacrificed by cervical dislocation. All animal experiments were performed in accordance with institutional, state and federal guidelines. Anti-IL-4 (11B11), anti-IL-12 (C17.18), anti-IFN-γ (AN17.18.24) antibodies purified from hybridoma supernatants at the Deutsches Rheuma-Forschungszentrum (DRFZ, Berlin, Germany) were used at a final concentration of 20 μg/mL. FITC-conjugated anti-CD4 (GK1.5, Miltenyi Biotec), Alexa Flour 405-conjugated anti-CD4 (GK1.5, purified and coupled at the DRFZ) and PE-conjugated anti-IL-12Rb2 (Clone 305719, R&D Systems, Minneapolis, MN, USA) were used for surface staining. FITC-conjugated anti-IL-17 (eBio17B7; eBioscience) Alexa Flour 647-conjugated anti-T-Bet (eBio4B10, eBioscience), PE-conjugated anti-RORγt (AFKJS-9,

eBioscience) were used for intracellular stainings. For STAT4 phosphorylation (pSTAT4) and phosphorylated STAT1 (pSTAT1) staining, we used an Alexa Fluor 647-conjugated pSTAT4 (pY693) and pSTAT1 (pY701) antibody, respectively (both from BD Biosciences).

Murine cell culture conditions and Th differentiation *in vitro*

Naïve $CD4^+CD62L^+$ cells from 6- to 8-wk-old BALB/c mice were isolated as described previously [46]. All cultures and assays were performed in RPMI supplemented with 10% fetal bovine serum (Sigma), 100 U/mL penicillium, 0.1 mg/mL streptomycin, 0.3 mg/mL glutamine (Invitrogen) and 10 μM β-mercaptoethanol at 37°C in 5% CO2. Cell cultures were set up at 10^6 cells/mL and stimulated in the presence of 1 μg/mL anti-CD3 (145-2C11, BD Biosciences), 1 μg/mL anti-CD28 (37.51) and irradiated (30 Gy) splenocytes isolated from BALB/c mice. For Th17 differentiation, cells were stimulated in the presence of TGF-β1 (1 ng/mL), IL-6 (20 ng/mL), IL-23 (20 ng/mL) (all from R&D Systems), anti-IL-4 and anti-IFN-γ. Where indicated, recombinant IL-12 (5 ng/mL; R&D Systems) and recombinant IFN-γ (10 ng/mL) were added. The IL-17-producing cells were isolated as described previously [17].

Isolation of cells according to IL-12Rβ2 expression

IL-12Rβ2 was stained according to the manufactor's instructions. IL-12Rβ2 high and low cells were sorted on a FACS Aria (BD Biosciences). Sorted cells were stimulated with 0.1 ng/mL PMA and 1 μg/mL ionomycin (Sigma-Aldrich) for 4 h. Briefly, 5 μg/mL Brefeldin A (Sigma Chemicals) was added after 1 h. The cells were stained for intracellular cytokines, as described previously [47]. Absolute cell numbers and cytokine expression were determined with a MACSQuant Analyzer (Miltenyi Biotec).

Isolation of IL-17- and IL-17/IFN-γ-secreting cells *ex vivo*

IL-17-producing cells were isolated *ex vivo* as described previously [17]. $CD4^+$ T cells were isolated with anti-CD4 magnetic beads (Miltenyi Biotec) and stimulated with 0.1 ng/mL PMA and 1 μg/mL ionomycin for 2 h. $IL\text{-}17^+$ $IFN\text{-}\gamma^+$ T cells were isolated by combining the IL-17 PE and IFN-γ APC Cytokine Secretion Assay & Detection Kits (Miltenyi Biotech) and sorting with a FACSAria (BD Biosciences).

Isolation of human IL-17-secreting cells *ex vivo*

PBMC were obtained from buffy coats of healthy donors (German Red Cross) by Ficoll-Hypaque gradient (Sigma-Aldrich). All experiments with human material were approved by the local ethics committee. $CD4^+$ T cells were isolated with anti-CD4 magnetic beads (Miltenyi Biotec) and stimulated with 0.1 ng/mL PMA and 1 μg/mL ionomycin for 4 h. $IL\text{-}17^+$ $IFN\text{-}\gamma^+$ $CD4^+$ T cells were stained by combining the human IL-17 and IFN-γ secretion assays (Miltenyi Biotech) and sorted with a FACSAria (BD Biosciences). For intracellular cytokine staining, anti-CD4-Alexa Fluor 405 (TT1), anti-IL-17A-Alexa Fluor 647 (eBioscience) and anti-IFN-γ-FITC (4SB3) were used. Isolated cells were cultured *in vitro* with 0.1 μg/mL anti-CD3 and 6 μg/mL anti-CD28 antibodies (BD Biosciences). Cytokines were used at either 10 ng/mL (IL-12 and IFN-γ, BD Biosciences), or 1000 U/mL (IL-2, Chiron). Neutralizing antibodies to IFN-γ (Peprotech) were used at 10 μg/mL.

EAE induction

EAE was induced in 8- to 12-wk-old C57BL/6 mice by subcutaneous injection of 200 μg myelin oligodendrocyte glycoprotein$_{35–55}$ peptide [48] (mevgwyrspfsrvvhlyrngk; synthesized by Dr. R. Volkmer, Charité, Berlin, Germany) emulsified in complete Freund's adjuvant (containing 1 mg/mL *Mycobacterium tuberculosis* H37RA, Sigma) together with i.v. administration of 200 ng pertussis toxin (Sigma) on days 0 and 2. Disease severity was assigned scores daily on a scale of 0–5 as follows: 0, no paralysis; 1, limp tail; 2, limp tail and partial hindleg paralysis; 3, complete hindleg paralysis; 4, tetraparesis and 5, moribund.

Flow cytometry

In brief, 5×10^6 cells/mL were stimulated in RPMI medium with PMA and Ionomycin (Sigma) for 4 h. In total, 5 μg/mL Brefeldin A was added after 1 h.

For intracellular T-bet and RORγt staining, we used the Foxp3 staining buffer set (eBioscience) according to the manufacturer's instructions. Mouse IgG1, κ (eBioscience) was used as Isotype Control for T-bet. $CD4^+CD62L^+$ were stained as negative control for RORγt. Intracellular IL-17 and IFN-γ in murine and human cells were stained under the same conditions. For pSTAT4 and pSTAT1 staining, we used Phosflow Lyse/Perm buffer and Perm Buffer III (BD Biosciences) according to the manufacturer's instruction. The cells were stimulated with IL-12 (10 ng/mL) for 30 min for pSTAT4 and with IFN-γ (10 ng/mL) for 15 min for pSTAT1 staining. Flow cytometric analysis was performed with LSRII and FACSCalibur, using CellQuest and FlowJo (Tree Star, Ashland, OR, USA) software.

RNA quantification

RNA preparation and cDNA synthesis was performed as described previously [46]. For normalization of murine and human cDNA, the transcripts for the housekeeping genes hypoxanthine guanine phosphoribosyl transferase and ubiquitin ligase H5 (UbcH5) were

quantified, respectively. Murine primer sets for the real-time PCR were as previously described [17] except for: murine IL-12Rβ2 F: 5′-CTgATCCTCCATTACAgAA-3′, IL-12Rβ2 R: 5′-CggAAgTAAC-gAATTgAgAA-3′, murine IFN-γ R2F: 5′-CCgAgTgAAgTAC-TggTTTC-3′ and IFN-γ R2R: 5′-gTgTTTggAgCACATCATC-3′. Human Primer Sets were as follows:

UbcH5 F: 5′-TCTTGACAATTCATTTCCCAACAG-3′, UbcH5 R 5′-TCAGGCACTAAAGGA TCATCTGG-3′, RORγt F: 5′-gAggAAgTCCATgTgggAgA-3′, RORγt R: 5′- TCCTAACCAgCAC-CACTTCC-3′, T-bet F: 5′-CCCCGGCTGCATATCG-3′ and T-bet R: 5′-ATCCTTTGGCAAAGGGGTTA-3′.

Acknowledgements: The authors thank Jenny Kirsch and Toralf Kaiser for excellent cell sorting. This work has been supported by the German Science Foundation (DFG SFB421, SFB618 and SFB TR52) and MUGEN-Network of Excellence. I. L. was funded by the Berlin-Brandenburg School of Regenerative Therapies (BSRT).

Conflict of interest: Anne Richter is a paid employee of Miltenyi Biotec. The other authors declare no financial or commercial conflict of interest.

References

1 **Khoruts, A., Miller, S. D. and Jenkins, M. K.,** Neuroantigen-specific Th2 cells are inefficient suppressors of experimental autoimmune encephalomyelitis induced by effector Th1 cells. *J. Immunol.* 1995. **155**: 5011–5017.

2 **Yang, Y., Weiner, J., Liu, Y., Smith, A. J., Huss, D. J., Winger, R., Peng, H. *et al.*,** T-bet is essential for encephalitogenicity of both Th1 and Th17 cells. *J. Exp. Med.* 2009. **206**: 1549–1564.

3 **Luger, D., Silver, P. B., Tang, J., Cua, D., Chen, Z., Iwakura, Y., Bowman, E. P. *et al.*,** Either a Th17 or a Th1 effector response can drive autoimmunity: conditions of disease induction affect dominant effector category. *J. Exp. Med.* 2008. **205**: 799–810.

4 **Tarrant, T. K., Silver, P. B., Chan, C.-C., Wiggert, B. and Caspi, R. R.,** Endogenous IL-12 is required for induction and expression of experimental autoimmune uveitis. *J. Immunol.* 1998. **161**: 122–127.

5 **Powrie, F., Leach, M. W., Mauze, S., Menon, S., Caddle, L. B. and Coffman, R. L.,** Inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RBhi CD4+T cells. *Immunity* 1994. **1**: 553–562.

6 **Cua, D. J., Sherlock, J., Chen, Y., Murphy, C. A., Joyce, B., Seymour, B., Lucian, L. *et al.*,** Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* 2003. **421**: 744–748.

7 **Yen, D., Cheung, J., Scheerens, H., Poulet, F., McClanahan, T., McKenzie, B., Kleinschek, M. A. *et al.*,** IL-23 is essential for T cell-mediated colitis and promotes inflammation *via* IL-17 and IL-6. *J. Clin. Invest.* 2006. **116**: 1310–1316.

8 **Kolls, J. K. and Linden, A.,** Interleukin-17 family members and inflammation. *Immunity* 2004. **21**: 467–476.

9 **Stout, R. D. and Bottomly, K.,** Antigen-specific activation of effector macrophages by IFN-gamma producing (TH1) T cell clones. Failure of IL-4-producing (TH2) T cell clones to activate effector function in macrophages. *J. Immunol. (Baltimore, MD)* 1989. **142**: 760–765.

10 **O'Connor, R. A., Prendergast, C. T., Sabatos, C. A., Lau, C. W., Leech, M. D., Wraith, D. C. and Anderton, S. M.,** Cutting edge: Th1 cells facilitate the entry of Th17 cells to the central nervous system during experimental autoimmune encephalomyelitis. *J. Immunol.* 2008. **181**: 3750–3754.

11 **Lees, J. R., Iwakura, Y. and Russell, J. H.,** Host T cells are the main producers of IL-17 within the central nervous system during initiation of experimental autoimmune encephalomyelitis induced by adoptive transfer of Th1 cell lines. *J. Immunol. (Baltimore, MDd)* 2008. **180**: 8066–8072.

12 **Infante-Duarte, C., Horton, H. F., Byrne, M. C. and Kamradt, T.,** Microbial lipopeptides induce the production of IL-17 in Th cells. *J. Immunol.* 2000. **165**: 6107–6115.

13 **Aarvak,T., Chabaud, M., Miossec, P. and Natvig, J. B.,** IL-17 is produced by some proinflammatory Th1/Th0 cells but not by Th2 cells. *J. Immunol.* 1999. **162**: 1246–1251.

14 **Annunziato, F., Cosmi, L., Santarlasci, V., Maggi, L., Liotta, F., Mazzinghi, B., Parente, E. *et al.*,** Phenotypic and functional features of human Th17 cells. *J. Exp. Med.* 2007. **204**: 1849–1861.

15 **Gorelik, L., Constant, S. and Flavell, R. A.,** Mechanism of transforming growth factor beta-induced inhibition of T helper type 1 differentiation. *J. Exp. Med.* 2002. **195**: 1499–1505.

16 **Harrington, L. E., Hatton, R. D., Mangan, P. R., Turner, H., Murphy, T. L., Murphy, K. M. and Weaver, C. T.,** Interleukin 17-producing CD4+effector T cells develop *via* a lineage distinct from the T helper type 1 and 2 lineages. *Nat. Immunol.* 2005. **6**: 1123–1132.

17 **Lexberg, M. H., Taubner, A., Forster, A., Albrecht, I., Richter, A., Kamradt, T., Radbruch, A. and Chang, H. D.,** Th memory for interleukin-17 expression is stable *in vivo*. *Eur. J. Immunol.* 2008. **38**: 2654–2664.

18 **Cosmi, L., Maggi, L., Santarlasci, V., Capone, M., Cardilicchia, E., Frosali, F., Querci, V. *et al.*,** Identification of a novel subset of human circulating memory CD4(+) T cells that produce both IL-17A and IL-4. *J. Allergy Clin. Immunol.* 2010. **125**: 222–230; e221–e224.

19 **Lee, Y. K., Turner, H., Maynard, C. L., Oliver, J. R., Chen, D., Elson, C. O. and Weaver, C. T.,** Late developmental plasticity in the T helper 17 lineage. *Immunity* 2009. **30**: 92–107.

20 **Schulz, E. G., Mariani, L., Radbruch, A. and Hofer, T.,** Sequential polarization and imprinting of type 1 T helper lymphocytes by interferon-gamma and interleukin-12. *Immunity* 2009. **30**: 673–683.

21 **van Rietschoten, J. G., Smits, H. H., van de Wetering, D., Westland, R., Verweij, C. L., den Hartog, M. T. and Wierenga, E. A.,** Silencer activity of NFATc2 in the interleukin-12 receptor beta 2 proximal promoter in human T helper cells. *J. Biol. Chem.* 2001. **276**: 34509–34516.

22 **Sanceau, J., Merlin, G. and Wietzerbin, J.,** Tumor necrosis factor-alpha and IL-6 up-regulate IFN-gamma receptor gene expression in human monocytic THP-1 cells by transcriptional and post-transcriptional mechanisms. *J. Immunol.* 1992. **149**: 1671–1675.

23 **Pernis, A., Gupta, S., Gollob, K. J., Garfein, E., Coffman, R. L., Schindler, C. and Rothman, P.,** Lack of interferon gamma receptor beta chain and the prevention of interferon gamma signaling in TH1 cells. *Science* 1995. **269**: 245–247.

24 **Afkarian, M., Sedy, J. R., Yang, J., Jacobson, N. G., Cereb, N., Yang, S. Y., Murphy, T. L. and Murphy, K. M.,** T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+T cells. *Nat. Immunol.* 2002. **3**: 549–557.

25 **Szabo, S. J., Dighe, A. S., Gubler, U. and Murphy, K. M.,** Regulation of the interleukin (IL)-12R beta 2 subunit expression in developing T helper 1 (Th1) and Th2 cells. *J. Exp. Med.* 1997. **185**: 817–824.

26 **Lohning, M., Richter, A. and Radbruch, A.,** Cytokine memory of T helper lymphocytes. *Adv. Immunol.* 2002. **80**: 115–181.

27 **Ansel, K. M., Lee, D. U. and Rao, A.,** An epigenetic view of helper T cell differentiation. *Nat. Immunol.* 2003. **4**: 616–623.

28 **Hwang, E. S., Szabo, S. J., Schwartzberg, P. L. and Glimcher, L. H.,** T helper cell fate specified by kinase-mediated interaction of T-bet with GATA-3. *Science* 2005. **307**: 430–433.

29 **Ouyang, W., Ranganath, S. H., Weindel, K., Bhattacharya, D., Murphy, T. L., Sha, W. C. and Murphy, K. M.,** Inhibition of Th1 development mediated by GATA-3 through an IL-4-independent mechanism. *Immunity* 1998. **9**: 745–755.

30 **Usui, T., Nishikomori, R., Kitani, A. and Strober, W.,** GATA-3 suppresses Th1 development by downregulation of Stat4 and not through effects on IL-12Rbeta2 chain or T-bet. *Immunity* 2003. **18**: 415–428.

31 **Grogan, J. L., Wang, Z. E., Stanley, S., Harmon, B., Loots, G. G., Rubin, E. M. and Locksley, R. M.,** Basal chromatin modification at the IL-4 gene in helper T cells. *J. Immunol.* 2003. **171**: 6672–6679.

32 **Murphy, E., Shibuya, K., Hosken, N., Openshaw, P., Maino, V., Davis, K., Murphy, K. and O'Garra, A.,** Reversibility of T helper 1 and 2 populations is lost after long-term stimulation. *J. Exp. Med.* 1996. **183**: 901–913.

33 **Annunziato, F., Cosmi, L. and Romagnani, S.,** Human and murine Th17. *Curr. Opin. HIV AIDS* 2010. **5**: 114–119.

34 **Mullen, A. C., High, F. A., Hutchins, A. S., Lee, H. W., Villarino, A. V., Livingston, D. M., Kung, A. L. *et al.*,** Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. *Science* 2001. **292**: 1907–1910.

35 **Toh, M.-L., Kawashima, M., Zrioual, S., Hot, A., Miossec, P. and Miossec, P.,** IL-17 inhibits human Th1 differentiation through IL-12R[beta]2 down-regulation. *Cytokine* 2009. **48**: 226.

36 **Nakae, S., Iwakura, Y., Suto, H. and Galli, S. J.,** Phenotypic differences between Th1 and Th17 cells and negative regulation of Th1 cell differentiation by IL-17. *J. Leukoc. Biol.* 2007. **81**: 1258–1268.

37 **Hegazy, A. N., Peine, M., Helmstetter, C., Panse, I., Frohlich, A., Bergthaler, A., Flatz, L. *et al.*,** Interferons direct Th2 cell reprogramming to generate a stable GATA-3(+)T-bet(+) cell subset with combined Th2 and Th1 cell functions. *Immunity* 2010. **32**: 116–128.

38 **Fili, L., Ferri, S., Guarna, F., Sampognaro, S., Manuelli, C., Liotta, F., Cosmi, L. *et al.*,** Redirection of allergen-specific TH2 responses by a modified adenine through Toll-like receptor 7 interaction and IL-12/IFN release. *J. Allergy Clin. Immunol.* 2006. **118**: 511–517.

39 **Lin, Y., Ritchea, S., Logar, A., Slight, S., Messmer, M., Rangel-Moreno, J., Guglani, L. *et al.*,** Interleukin-17 is required for T helper 1 cell immunity and host resistance to the intracellular pathogen *Francisella tularensis*. *Immunity* 2009. **31**: 799–810.

40 **Suryani, S. and Sutton, I.,** An interferon-gamma-producing Th1 subset is the major source of IL-17 in experimental autoimmune encephalitis. *J. Neuroimmunol.* 2007. **183**: 96–103.

41 **Lim, H. W., Lee, J., Hillsamer, P. and Kim, C. H.,** Human Th17 cells share major trafficking receptors with both polarized effector T cells and FOXP3+regulatory T cells. *J. Immunol.* 2008. **180**: 122–129.

42 **Annunziato, F., Cosmi, L., Liotta, F., Maggi, E. and Romagnani, S.,** The phenotype of human Th17 cells and their precursors, the cytokines that mediate their differentiation and the role of Th17 cells in inflammation. *Int. Immunol.* 2008. **20**: 1361–1368.

43 **Janke, M., Peine, M., Nass, A., Morawietz, L., Hamann, A. and Scheffold, A.,** *In vitro*-induced Th17 cells fail to induce inflammation *in vivo* and show an impaired migration into inflamed sites. *Eur. J. Immunol.* 2010. **40**: 1089–1098.

44 **Boniface, K., Blumenschein, W. M., Brovont-Porth, K., McGeachy, M. J., Basham, B., Desai, B., Pierce, R. *et al.*,** Human Th17 cells comprise heterogeneous subsets including IFN-gamma-producing cells with distinct properties from the Th1 lineage. *J. Immunol.* 2010. **185**: 679–687.

45 **Kebir, H., Ifergan, I., Alvarez, J. I., Bernard, M., Poirier, J., Arbour, N., Duquette, P. and Prat, A.,** Preferential recruitment of interferon-gamma-expressing T H 17 cells in multiple sclerosis. *Ann. Neurol.* 2009. **66**: 390–402.

46 **Chang, H. D., Helbig, C., Tykocinski, L., Kreher, S., Koeck, J., Niesner, U. and Radbruch, A.,** Expression of IL-10 in Th memory lymphocytes is conditional on IL-12 or IL-4, unless the IL-10 gene is imprinted by GATA-3. *Eur. J. Immunol.* 2007. **37**: 807–817.

47 **Murphy, K. M., Heimberger, A. B. and Loh, D. Y.,** Induction by antigen of intrathymic apoptosis of CD4+CD8+TCRlo thymocytes *in vivo*. *Science* 1990. **250**: 1720–1723.

48 **Brustle, A., Heink, S., Huber, M., Rosenplanter, C., Stadelmann, C., Yu, P., Arpaia, E. *et al.*,** The development of inflammatory T(H)-17 cells requires interferon-regulatory factor 4. *Nat. Immunol.* 2007. **8**: 958–966.

Abbreviations: **IFN-γ R2:** IFN-γ receptor 2 · **pSTAT1:** phosphorylated STAT1 · **pSTAT4:** STAT4 phosphorylation · **RORγt:** related orphan receptor γt · **UbcH5:** ubiquitin ligase H5

Full correspondence: Dr. Hyun-Dong Chang, Deutsches Rheuma-Forschungszentrum Berlin, a Leibniz Institute, Charitéplatz 1, 10117 Berlin, Germany
Fax+49-30-28460-603
e-mail: chang@drfz.de

Additional correspondence: Prof. Andreas Radbruch, Deutsches Rheuma-Forschungszentrum Berlin, a Leibniz Institute, Charitéplatz 1, 10117 Berlin, Germany
e-mail: radbruch@drfz.de

Received: 31/3/2010
Revised: 20/7/2010
Accepted: 31/8/2010
Accepted article online: 21/9/2010

3.3 Th-Zellen exprimieren Zytokine nach dem Alles-oder-nichts-Prinzip in Abhängigkeit von NFAT

Köck J, Kreher S, Lehmann K, Riedel R, Bardua M, Lischke T, Jargosch M, Haftmann C, Bendfeldt H, Hatam F, Mashreghi MF, Baumgrass R, Radbruch A, **Chang HD**. 2014. Nuclear factor of activated T cells regulates the expression of interleukin-4 in Th2 cells in an all-or-none fashion. *J Biol Chem.* 289(39):26752-61.

Zimmermann J, Radbruch A, **Chang HD**. 2015. A Ca(2+) concentration of 1.5 mM, as present in IMDM but not in RPMI, is critical for maximal response of Th cells to PMA/ionomycin. *Eur J Immunol.* 45(4):1270-3.

Th-Zellen werden anhand ihrer Zytokinexpression nach Restimulation, in der Regel mit PMA und Ionomycin, in unterschiedliche Kategorien klassifiziert, wie z.B. Th1 bei IFNγ-Expression, Th2 bei IL-4-Expression oder Th17 bei IL-17-Expression. Auf Einzelzellebene exprimiert jedoch nicht jede Th-Zelle bei einer bestimmten Restimulation die Zytokine, deren Gene epigenetisch geprägt sind. So kann eine Th2-Zelle bei einer Restimulation IL-4 machen und bei der nächsten Restimulation nicht. Diese scheinbare Stochastik bei der Zytokinexpression macht nicht nur die funktionelle Einteilung von Th-Zellen anhand ihrer Zytokinexpression problematisch sondern wirft auch die Frage auf, wie eine individuelle Th-Zelle ihre Zytokin-Expression reguliert.
Wir haben anhand von Th2-Zellen die molekularen Mechanismen untersucht, die der Zytokinexpression nach T-Zellrezeptorstimulation zugrunde liegen, um zu verstehen wie die Entscheidung getroffen wird, ob eine bestimmte Th-Zelle ein bestimmtes Zytokin exprimiert oder nicht. Mittels Chromatin-Immunprezipitation (ChIP) konnten wir zeigen, dass die Rekrutierung und Bindung von den Transkriptionsfaktoren NF-κB, c-Maf, CBP/p300, GATA-3 und NFAT an den Promoter des *Il4*-Gens nur nach T-Zellrezeptorstimulation erfolgt. Die Bindung der Transkriptionsfaktoren stimmt zeitlich mit der Expression der IL-4-mRNA überein, d.h. sie binden gleichzeitig, wahrscheinlich als Transkriptionsfaktorkomplex, um die *Il4*-Expression anzuschalten. Der Transkriptionsfaktorkomplex war nur in Th2-Zellen nachweisbar, die nach Restimulation IL-4 exprimierten und nicht in IL-4-Nicht-Produzenten. In Th2-Zellen konnte die IL-4 Expression mittels NFAT Blockade inhibiert werden. Interessanterweise, wurde durch NFAT-Blockade auch die Bindung aller anderen Komponenten des Transkriptionsfaktorkomplexes an

den *Il4*-Promoter inhibiert. Dies deutet darauf hin, dass NFAT ein kritischer Faktor für den Zusammenbau des Transkriptionsfaktorkomplexes an das *Il4*-Gen ist. Eine graduelle Inhibition von NFAT führte zu einer graduellen Abnahme in der Frequenz der IL-4-Produzenten unter den Th2-Zellen aber nicht in der Menge an IL-4 pro Zelle. IL-4 wird also nach dem Alles-oder-nichts-Prinzip in Abhängigkeit von NFAT exprimiert. Mittels bildgebender Durchflusszytometrie konnten wir zeigen, dass in allen Th2-Zellen, die IL-4 produzieren, NFAT auch im Zellkern vorliegt. NFAT ist demnach der kritische Schalter, der graduelle T-Zellrezeptorsignale in eine binäre Entscheidung IL-4 zu produzieren oder nicht umwandelt.
Demnach wird die Stärke der Th-Zellantwort über die Anzahl der Th-Zellen, die reagieren, an unterschiedliche Antigenmengen über den NFAT-Schalter angepasst. Dadurch ist das Immunsystem in der Lage auf sehr geringe Mengen an Antigen mit wenigen Zellen zu reagieren.

Anhand unserer Ergebnisse haben wir auch die Bedingungen optimiert in denen die Zytokinexpression von Th- Zellen nach Restimulation mit PMA und Ionomycin abgefragt wird. Eine Restimulation mit PMA und Ionomycin dient in der Regel dazu das maximale Potential einer Zelle abzurufen. Wir konnten zeigen, dass in RPMI-Medium, welches häufig zur Restimulation genutzt wird, Kalzium limitierend ist und Calcineurin nicht optimal aktiviert werden kann. Durch Zugabe von ausreichenden Mengen an Kalzium bzw. durch die Nutzung von Medien mit höheren Kalzium-Konzentrationen, wie z.B. IMDM-Medium, kann sichergestellt werden, dass die Bedingungen für die NFAT Aktivierung durch das Kalzium-abhängige Calcineurin optimal sind.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 289, NO. 39, pp. 26752–26761, September 26, 2014

Nuclear Factor of Activated T Cells Regulates the Expression of Interleukin-4 in Th2 Cells in an All-or-none Fashion*

Received for publication, June 10, 2014, and in revised form, July 14, 2014 Published, JBC Papers in Press, July 17, 2014, DOI 10.1074/jbc.M114.587865

Juliana Köck, Stephan Kreher, Katrin Lehmann, René Riedel, Markus Bardua, Timo Lischke, Manja Jargosch, Claudia Haftmann, Hanna Bendfeldt, Farahnaz Hatam, Mir-Farzin Mashreghi, Ria Baumgrass, Andreas Radbruch, and Hyun-Dong Chang[1]

From the German Rheumatism Research Center Berlin, a Leibniz Institute, Charitéplatz 1, 10117 Berlin, Germany

Background: Not every T helper type 2 (Th2) lymphocyte imprinted to express interleukin-4 (IL-4) does so when activated.
Results: Preventing nuclear translocation of the nuclear factor of activated T cells (NFAT) reduces the number of Th2 lymphocytes reexpressing IL-4.
Conclusion: NFAT is the limiting factor determining digital IL-4 expression in Th2 lymphocytes.
Significance: This might help us to understand the regulation of immunopathology in allergy and asthma.

Th2 memory lymphocytes have imprinted their *Il4* genes epigenetically for expression in dependence of T cell receptor restimulation. However, in a given restimulation, not all Th cells with a memory for IL-4 expression express IL-4. Here, we show that in reactivated Th2 cells, the transcription factors NFATc2, NF-kB p65, c-Maf, p300, Brg1, STAT6, and GATA-3 assemble at the *Il4* promoter in Th2 cells expressing IL-4 but not in Th2 cells not expressing it. NFATc2 is critical for assembly of this transcription factor complex. Because NFATc2 translocation into the nucleus occurs in an all-or-none fashion, dependent on complete dephosphorylation by calcineurin, NFATc2 controls the frequencies of cells reexpressing *Il4*, translates analog differences in T cell receptor stimulation into a digital decision for *Il4* reexpression, and instructs all reexpressing cells to express the same amount of IL-4. This analog-to-digital conversion may be critical for the immune system to respond to low concentrations of antigens.

T helper (Th)[2] lymphocytes regulate immune responses by expression of cytokines instructing themselves and other cells to qualified reactions. Different cytokines are expressed by different lineages of Th cells, to adapt immune responses to the diversity of pathogens. Differentiation of activated Th cells into a particular lineage is induced by costimulatory signals and determined by lineage-determining transcription factors. T-bet, GATA-3, and RORγt determine the Th1, Th2, and Th17 lineages, respectively (reviewed in Ref. 1). Lineage-determining master transcription factors are both essential and sufficient for the differentiation of Th cells into a distinct lineage.

Expression of GATA-3 is under the control of a distal promoter responsive to T cell receptor stimulation, and of a proximal promoter responsive to GATA-3 itself and to STAT6, the signal transducer of the receptor for the cytokine interleukin-4 (IL-4) (2, 3). Once induced, GATA-3 expression is stabilized by a positive feedback loop (4, 5). IL-4 is the signature cytokine of Th2 lymphocytes. GATA-3 is critical for the epigenetic imprinting of IL-4 for reexpression in reactivated Th2 cells (6). GATA-3 binds to a conserved intronic regulatory element (CIRE) in the first intron of the *Il4* gene and induces its demethylation, which correlates with its imprinting for reexpression (7). GATA-3 has been described to block methyl CpG binding domain protein-2, which links DNA methylation to silent chromatin (8). Other regulatory elements of the *Il4* gene include the hypersensitivity site Va (9) important for lineage-specific binding of NFAT to the *Il4* locus and a locus control region (LCR) within the *Rad50* gene upstream of the *Il4* gene (10). In addition to GATA-3, some other transcription factors participating in the transcriptional control of the *Il4* gene such as STAT6 (11), Brahma-related gene 1 (Brg1) (12), and Creb-binding protein CBP/p300 (13) have the ability to recruit histone acetyltransferases and block DNA methyltransferases. Th2 reexpress their imprinted *Il4* gene upon restimulation of the T cell receptor (14), however, not all of them.

A substantial fraction of Th2 cells will not reexpress *Il4* in a given restimulation. This is not due to an insufficient imprinting of the gene because the very same cells can reexpress the *Il4* gene in later restimulations, with similar efficacy as their sister cells in the original restimulation (15). The reason for the failure of a Th2 cell to reexpress *Il4* in a given restimulation could be either a rate-limiting, stochastic availablility of transcription factors controlling *Il4* expression in the nucleus, leading to monoallelic expression of the *Il4* gene, with some cells not expressing it at all (16). Alternatively, one transcription factor could control the assembly of the *Il4* transcriptional complex in an all-or-none fashion. This has been demonstrated for the control of reexpression of the cytokine IL-2, which is dependent

* This work was supported by the German Research Council (Deutsche Forschungsgemeinschaft SFB618 and SFB TRR52), the European Research Council Advanced Grants ERC-2010-AdG_20100317 and 268987, and the German Ministry of Education and Research Grant "IMPAM" (01EC1008B) and e:Bio-Innovationswettbewerb Systembiologie."
⌘ *Author's Choice*—Final version full access.
[1] To whom correspondence should be addressed: German Rheumatism Research Center Berlin, a Leibniz Institute, Charitéplatz 1, 10117 Berlin, Germany. Tel.: 49-0-30-28460-683; Fax: 49-0-30-28460-603; E-mail: chang@drfz.de.
[2] The abbreviations used are: Th, T helper; CsA, cyclosporin A; BTP1, 3,5-bis-trifluoromethyl pyrazole; CIRE, conserved intronic regulatory element; HSS, hypersensitivity site; NFAT, nuclear factor of activated T cells; STAT, signal transducer and activator of transcription; LCR, locus control region; PMA, phorbol 12-myristate 13-acetate; TCR, T cell receptor.

on translocation of NFATc2 into the nucleus (17). This translocation is dependent on complete dephosphorylation of NFATc2 at 13 positions by calcineurin (18), a reaction of second order, resulting in an all-or-none translocation of NFATc2 into the nucleus in individual Th2 cells. In addition, dephosphorylation at serine residues in the N-terminal transactivation domain is required for transcriptional activation.

Here, we show that in restimulated Th2 cells NFATc2 controls the reexpression of *Il4* in an all-or-none fashion. NFATc2 translocation into the nucleus is required for assembly of the transcription factor complex at the *Il4* promoter, which occurs only in IL-4-expressing Th2 cells, upon restimulation of the T cell receptor (TCR). Modulation of TCR signaling strength by graded inhibition of NFAT results in decreasing frequencies of IL-4-expressing Th2 cells. The amount of IL-4 produced by expressing cells is not affected. Thus, in Th2 cells NFAT serves as a molecular switch that translates graded differences in TCR signal strength into a digital decision to express IL-4 or not.

EXPERIMENTAL PROCEDURES

Mice—BALB/c, C57BL/6, OVA-TCRtg/tg DO11.10 (kind gift of Dennis Y. Loh and Kenneth Murphy, Washington University School of Medicine, St. Louis, MO), and OT-II mice were bred under specific pathogen-free conditions in our animal facility. The mice were sacrificed by cervical dislocation. All animal experiments were performed in accordance with institutional, state, and federal guidelines.

Antibodies—All antibodies used in these experiments were either conjugated in-house or purchased as indicated. Anti-IL-4 (11B11), anti-IL-12 (C17.18), anti-IFNγ (AN17.18.24), and anti-CD4 (GK1.5) antibodies were purified from hybridoma supernatants at the German Rheumatism Research Center and used at 10 μg/ml final concentration. FITC-conjugated anti-IFNγ (AN18.17.24; BD Pharmingen, Heidelberg, Germany), phycoerythrin-conjugated anti-IL-4 (11B11; BD Pharmingen, and BVD4–1D11; Miltenyi Biotec, Bergisch Gladbach, Germany) were used for intracellular cytokine staining. For the chromatin immunoprecipitation, the following antibodies were used: anti-c-MAF, anti-RNA polymerase II, anti-p300 and anti-STAT6 (polyclonal rabbit IgG; Santa Cruz Biotechnology, Heidelberg, Germany), anti-NFATc2 and -NFATc1 (polyclonal rabbit IgG; ImmunoGlobe Antikörpertechnik GmbH, Himmelstadt, Germany), anti-GATA-3 (mouse monoclonal; Santa Cruz Biotechnology), anti-NF-kB (polyclonal goat IgG; Santa Cruz Biotechnology), anti-Brg1 (rabbit antiserum; Merck-Millipore, Darmstadt, Germany). For the image cytometry, anti-NFATc2 (rabbit monoclonal, clone D4B1, Cell Signaling Technology, Leiden, The Netherlands) and donkey anti-rabbit IgG (coupled to Alexa Fluor 647, Molecular probes A31573, Darmstadt, Germany) were used.

In Vitro Th Cell Differentiation—$CD4^+CD62L^+$ cells from 6–8-week-old DO11.10 or OT-II mice were isolated and differentiated into Th1 and Th2 lineages as described (19). In short, for Th1 differentiation, cells were stimulated in the presence of recombinant IL-12 (5 ng/ml; R&D Systems, Wiesbaden, Germany) and anti-IL4 (11B11) antibody for 6 days. For Th2 differentiation, cells were stimulated in the presence of IL-4 (100 ng/ml, culture supernatant of HEK293T cells transfected with murine IL-4 cDNA), anti-IL12 (C17.8), and anti-IFNγ (AN18.17.24) antibodies.

Isolation of Viable IL-4 Secreting Cells—Viable IL-4 secreting cells were isolated as described previously (14). The secreted IL-4 was detected with an anti-IL4 phycoerythrin-conjugated antibody (Miltenyi Biotec). The IL-4 producing cells and the IL-4 non-producing cells were separated by MACS using anti-phycoerythrin microbeads (Miltenyi Biotec). After sorting, the purity of the sort was confirmed with a FACSCalibur (BD Biosciences).

Chromatin Immunoprecipitation—Cells were harvested at the indicated time points and fixed with 1% formaldehyde for 10 min at room temperature. The chromatin immunoprecipitation assay was performed as described previously (19). The following primers were used: *Il4* promoter up, 5′-GGCCCAGAATAACTGACAATCT-3′ and *Il4* promoter down, 5′-GCAATGCTGGCAGAGGTC-3′; CIRE up, 5′-CACTTGAGAGAGATCATCGG-3′ and CIRE down, 5′-CCACCTCTCTAGCAACTCAG-3′; *Il4* hypersensitivity site (HSS) Va up, 5′-TTGGGTTCTCAGTCCAACAGA-3′ and *Il4* HSS Va up, 5′-CCAGGGCACTTAAACATTGC-3′; CNS1 up, 5′-GGGAGTTTCTTAGGCCCTCT-3′ and CNS1 down, 5′-CCCCCTCTCACTGTGAAAAC-3′; LCRRad50 up, 5′-CCACACACTGGGATGTGTAGCTCA-3′ and LCRRad50 down, 5′-AGACCCAGCTCCTCAGAAGGTAGT-3′; and *Ifng* promoter up, 5′-TTTCAGAGAATCCCACAAGAATG-3′ and *Ifng* promoter down, 5′-TCGGGATTACGTATTTTCACAAG-3′.

Intracellular Cytokine Staining—For intracellular cytokine staining, the cultured Th cells are harvested and restimulated with 10 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1 μg/ml ionomycin (Sigma) for 2 h followed by additional 2 h in the presence of 5 μg/ml brefeldin A (Sigma Chemicals). The cells were washed with PBS and fixed with 2% formaldehyde (Merck-Millipore). The cell membrane was permeabilized with 0.5% sapinon in PBS/BSA for intracellular staining. The staining was measured with a FACSCalibur (BD Biosciences) or with a MACSQuant Analyzer (Miltenyi Biotec), and the data were analyzed using FlowJo (Treestar). For calcineurin/NFAT inhibition, cyclosporin A, BTP1 (3,5-bistrifluoromethyl pyrazole), or 11R-VIVIT (Calbiochem) was given to the cells at the indicated concentrations 15 min prior to addition of PMA and ionomycin. For siRNA-mediated inhibition of NFATc2, Accell siRNA (A-054724-16; Dharmacon, Lafayette, CO) specific for NFATc2 and control siRNA (D-001910-03-05; Dharmacon) were used as described in Ref. 20. Knockdown efficiency was determined by RT-PCR using the following primers: NFATc2 up, 5′-GGTTGCTCCTCTGCCCGCAG-3′ and NFATc2 down, 5′-TTGGAGGGGATCCCGCAGGG-3′.

Image Cytometry—Th2 cells were harvested and restimulated with PMA/ionomycin for 3 h in the presence of 7 nM cyclosporin A (CsA). The cells were fixed with 1% formaldehyde. Cytokine staining was performed in 0.5% saponin. NFATc2 staining was done in Foxp3 staining buffer (eBioscience). Staining was analyzed using the Imagestream MKII (Amnis Merck-Millipore). For nuclear staining, DAPI was added before analysis. Data analysis was performed using the IDEAS software (Amnis). Nuclear localization of NFATc2 was determined by "similarity" of NFATc2 and DAPI on a per cell

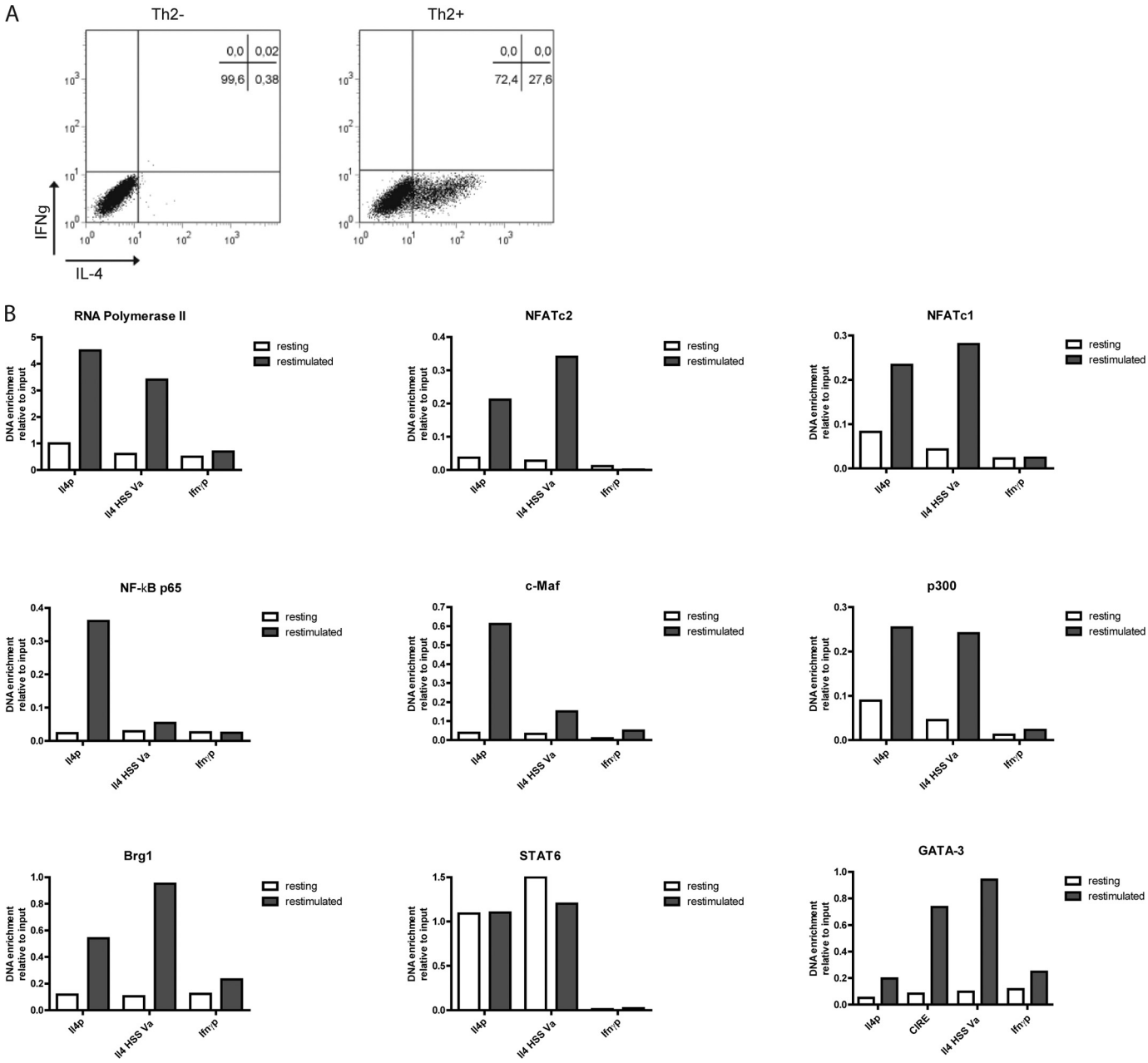


FIGURE 1. **The binding of transcription factors to the Il4 locus is dependent on restimulation.** *A*, Th2 cells polarized in the presence of recombinant IL-4, anti-IL12, and anti-IFNγ antibodies for 12 days were restimulated with PMA and ionomycin for 3 h and then stained intracellularly for IL-4 and IFNγ expression. *B*, the Th2 cells were either fixed in a resting state or after restimulation. ChIP was performed against RNA polymerase II, NFATc2, NFATc1, NF-κBp65, c-maf, CBP/p300, Brg1, STAT6, and GATA-3 and probed for binding regions in the *Il4* gene. Binding to the *Ifn*γ promoter was used as negative control. Representative result of three independent experiments.

basis. The similarity score is a log-transformed Pearson's correlation coefficient of the pixel values of the DAPI and NFATc2 staining.

RESULTS

Assembly of the Activating Transcription Factor Complex at the Il4 Locus in Th2 Cells Is Dependent on T Cell Receptor Stimulation—We generated Th2 cells by stimulating naive $CD4^+CD62L^+$ T cells from ovalbumin-specific T cell receptor transgenic DO11.10 mice with $OVA_{323–339}$ for 12 days in the presence of recombinant IL-4 and antibodies blocking IFN-γ and IL-12. On day 6, fresh antigen-presenting cells, antigen, IL-4, and antibodies were added. On day 12, the resting Th2 cells were either fixed directly or restimulated with PMA and ionomycin for 3 h and used for chromatin immunoprecipitation (ChIP) to assay for binding of RNA polymerase II and the transcription factors NFATc2, NFATc1, NF-κB p65, c-Maf, p300, Brg1, STAT6, and GATA-3 to the *Il4* promoter and the *Il4* HSS Va. Binding of GATA-3 to CIRE was also assayed. Binding to the *Ifn*γ promoter was used as negative control. In resting Th2 cells, in which no IL-4 is detectable by intracellular cytokine staining (Fig. 1*A*), none of the transcription factors with the exception of STAT6 bound to any of the regions tested (Fig. 1*B*). STAT6 bound to both the *Il4* promoter and *Il4* HSS Va to

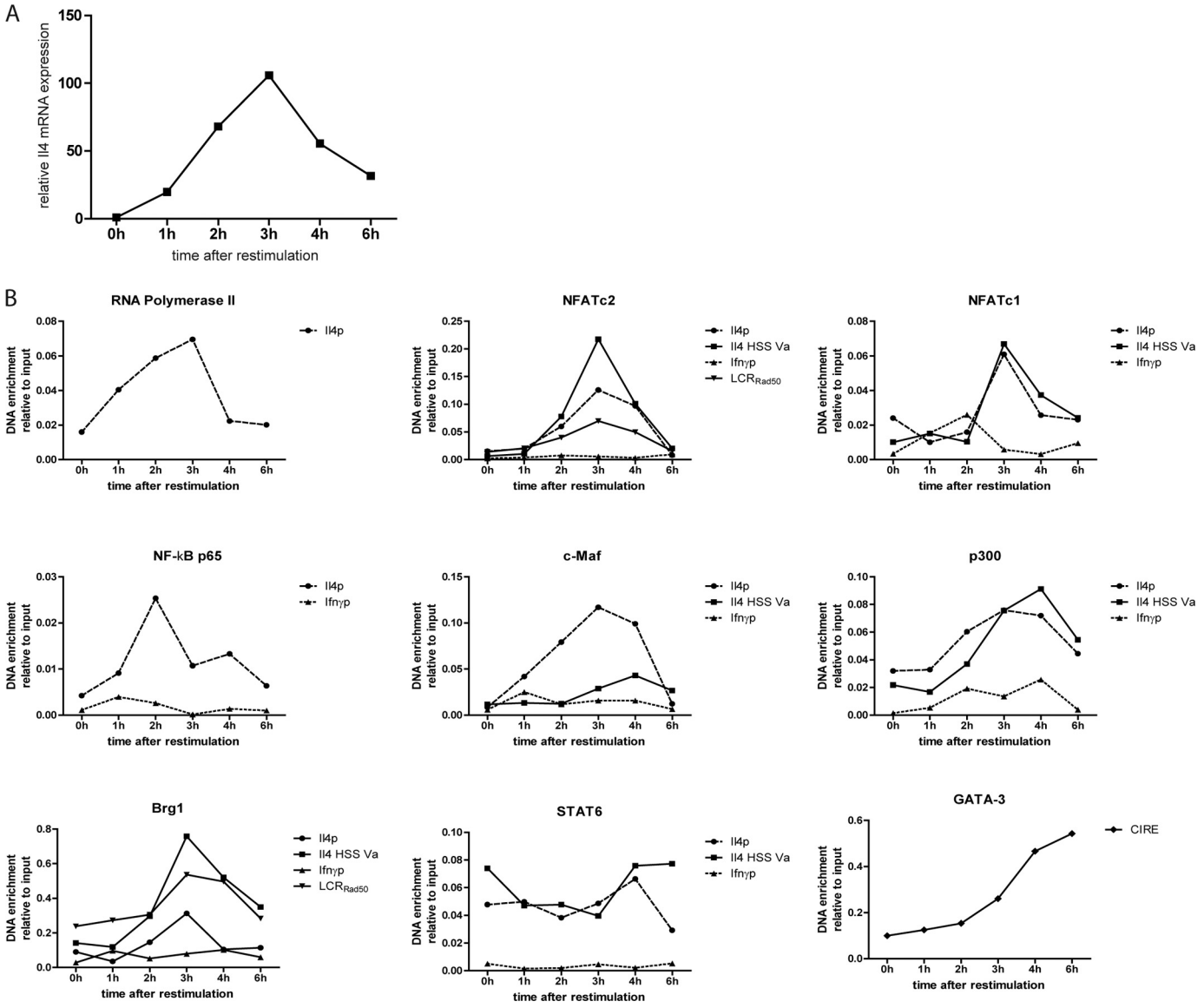


FIGURE 2. **The binding of transcription factors to the Il4 locus occurs in a coordinated fashion.** *A*, Th2 cells polarized for 12 days were restimulated, and the Il4 mRNA expression was quantified every hour for 6 h. *B*, the Th2 cells were restimulated, and aliquot was fixed every hour. ChIP was performed against the indicated transcription factors to determine the binding kinetic of the factors to selected regulatory regions in the *Il4* locus. Binding to the *Ifnγ* promoter was used as negative control. Shown are the representative results of two independent experiments.

the same degree in unstimulated and restimulated cells. In restimulated Th2 cells, all transcription factors analyzed bound to the *Il4* promoter, except for GATA-3, which does not have a binding site there. NFATc2, NFATc1, p300, Brg1, and GATA-3 also bound to the *Il4* HSS Va. GATA-3 also bound to the CIRE. Thus, the assembly of TCR dependent and independent transcription factors at the *Il4* gene of Th2 cells is dependent on TCR stimulation, *i.e.* the activation of one or more TCR-dependent transcription factors.

Coordinated Assembly of Transcription Factors at the Il4 Locus—To determine the kinetics of transcription factor assembly to the *Il4* locus, we fixed *in vitro*-generated Th2 cells before restimulation (0 h) and 1, 2, 3, 4, and 6 h following restimulation with PMA/ionomycin. Activated Th2 cells showed detectable levels of IL-4 mRNA already after 1 h and reached a maximum expression at 3 h, after which it declined again (Fig. 2*A*). IL-4 protein expression follows a similar time course (14). Binding of RNA polymerase II to the *Il4* promoter reached a maximum after 3h, with an abrupt drop to baseline levels after 4 h (Fig. 2*B*). The transcription factors NFATc2, NFATc1, c-MAF, p300, and Brg1 reached their maximal binding to the *Il4* promoter after 3 h. The transcription factors analyzed bound with similar kinetics also to the *Il4* HSS Va. No significant binding to the *Ifnγ* promoter could be detected at any time point. STAT6 bound to the *Il4* promoter and *Il4* HSS Va at all time points tested. NFATc2 and Brg1 also bound to the locus control region, located in the *Rad50* gene (LCR_{Rad50}), reaching maximum binding after 3 h. GATA-3 binding to the CIRE increases after PMA/ionomycin restimulation and continues to increase until 6 h after the onset of restimulation, the end of the period of observation. The kinetics of transcription factor assembly at the *Il4* gene indicates the coordinated, interdependent assembly of all factors.

The Transcription Factor Complex Assembles at the Il4 Locus in IL-4 Expressing Th2 Cells but Not in IL-4-non-expressing Th2 Cells—Th2 cells were restimulated with PMA/ionomycin, and the IL-4 expression was determined (Fig. 3*A*). Of the Th2 cells, 55% expressed IL-4, whereas 45% did not express any detectable IL-4. IL-4-expressing and non-expressing Th2 cells were physically separated to >95% purity, using the IL-4 cytokine secretion assay, which we had developed earlier (14). IL-4 protein expression correlated with IL-4 mRNA expression as analyzed by quantitative PCR in the sorted populations (Fig. 3*B*). Both the IL-4 expressing and non-expressing Th cells expressed equal amounts of GATA-3, qualifying both fractions as *bona fide* Th2 cells (Fig. 3*C*).

Relative to the binding of the transcription factors to the *Ifn*γ promoter, no significant binding to any of the *Il4* gene regions analyzed from IL-4-non-expressing cells was observed for NF-κB p65, c-Maf, and RNA polymerase II. NFATc2, NFATc1, p300, and Brg1 did not bind to the promoter and CIRE regions, whereas STAT6 and GATA-3 did (Fig. 3*D*). STAT6 and GATA-3 also bound to the CIRE, LCR_{Rad50}, and HSS Va regions of Th2 cells not expressing IL-4 (Fig. 3*D*). For all regions of *Il4* analyzed, significantly more RNA polymerase II, NFATc2, NFATc1, NF-κB p65, c-Maf, p300, and Brg1 was detected in IL-4 expressing *versus* non-expressing cells. Thus, the transcription factor complex, with the exception of GATA-3 and STAT6, efficiently assembles only at *Il4* genes of IL-4-expressing Th2 cells.

Calcineurin Digitalizes IL-4 Expression in Th2 Cells—Naive DO11.10 TCR transgenic $CD4^+$ Th cells were stimulated under Th2-polarizing conditions for 12 days and then restimulated with PMA/ionomycin. IL-4 expression was assessed by intracellular cytokine staining showing that 34% of the Th2 cells reexpressed IL-4. When the NFATc2 dephosphorylation by calcineurin was selectively blocked by 25 nM of the specific inhibitor BTP1, a 3,5-bistrifluoromethyl pyrazole derivative (21), IL-4 reexpression was completely blocked (Fig. 4*A*). In those cells, binding of RNA polymerase II, p300, NFATc2, c-Maf, Brg1, and NF-κB p65 to the *Il4* promoter, 3 h after restimulation (Fig. 4*B*), was decreased to levels observed for IL-4-non-expressing Th2 cells (Fig. 3*D*). This shows that the dephosphorylation of NFATc2 by calcineurin is critical for the assembly of a transcriptional activator complex at the *Il4* gene.

Calcineurin, thus, translates graded differences in TCR signaling into an all-or-none expression of *Il4* of restimulated Th2 cells. This became evident when calcineurin was inhibited by CsA in different concentrations. Increasing CsA concentrations resulted in dose-dependent, decreased frequencies of IL-4 expressing Th2 cells following restimulation with PMA/ionomycin (Fig. 4*C*) or anti-CD3/CD28 antibodies (data not shown). However, the amount of IL-4 expressed by individual IL-4-expressing cells remained the same. As CsA has been described to also affect NF-κB activation (22), NFAT dephosphorylation was also blocked by the specific peptide inhibitor 11R-VIVIT (Fig. 4*D*) (23) and by specific siRNA targeting NFATc2 (Fig. 4*E*). Specific inhibition of either NFAT desphosphorylation by 11R-VIVIT or knockdown of NFATc2 itself by siRNA resulted in the reduction of the frequency of IL-4-expressing Th2 cells but not the amount of IL-4 expressed per cell.

IL-4 Expression Correlates with NFATc2 Nuclear Translocation—To visualize the nuclear translocation of NFATc2 in Th2 cells on the single cell level, *in vitro*-generated Th2 cells were restimulated with PMA/ionomycin in the presence of 7 nM CsA and stained for NFATc2 and IL-4. The cells were analyzed by image cytometry. Among all IL-4-expressing Th2 cells (Fig. 5*A*), NFATc2 showed nuclear localization which was defined by a high similarity score representing the correlation coefficient between the NFATc2 fluorescent signal and the nuclear DAPI fluorescent signal (Fig. 5, *B* and *C*). Th2 cells that did not reexpress IL-4 showed a bimodal distribution having either only cytoplasmic or only nuclear NFATc2 (Fig. 4, *B* and *C*). Taken together, our data indicate that in individual Th2 cells, calcineurin, by cooperative activating dephosphorylation of NFATc2, digitalizes graded differences in TCR signaling into all-or-none decisions to express IL-4.

DISCUSSION

Here, we show that in the TCR signaling cascade, calcineurin, by cooperative dephosphorylation of NFATc2, translates differences of signaling strength in individual, restimulated Th2 lymphocytes into an all-or-none decision to express or not the signature cytokine IL-4. NFAT is required to assemble at the *Il4* gene a transcription factor complex containing GATA-3, RNA polymerase II, NFATc2, NFATc1, NF-κB p65, c-Maf, CBP/p300, Brg1, and STAT6. Of these, only STAT6 and GATA-3 can bind in the absence of NFAT in Th2 cells not expressing IL-4.

Th2 lymphocytes are imprinted epigenetically by DNA demethylation and histone modification of the *Il4* gene (7, 24) and transcriptionally by expression of the lineage-determining transcription factor GATA-3 (5, 25), to express the signature cytokine IL-4 when restimulated by antigen. Surprisingly, and as noted early on, not all Th2 cells express IL-4 in a given restimulation (15). This is not a matter of lack of competence, as Th2 cells not expressing IL-4 in a given restimulation can express IL-4 in a subsequent restimulation at frequencies equal to cells that had expressed IL-4 (15). It has been speculated that the infidelity of IL-4 reexpression by individual Th2 cells might be due to stochastic, monoallelic expression of the *Il4* gene, with some cells not expressing it at all. It remained unclear, whether accessibility of the *Il4* gene (26–28) or availability of the transcription factors necessary for expression were the rate-limiting determinants. Moreover, in these studies, monoallelic expression of *Il4* was analyzed for genetically modified T lymphocytes that had one *Il4* allele marked by knock-in of a reporter gene, either green fluorescent protein (*Gfp*) (15) or *CD2* (29). For the *Gfp* knock-in Th lymphocytes, we have shown previously that the genetic insertion had replaced the GATA-3 binding site CIRE, disabling the epigenetic imprinting of the modified *Il4* allele (7). For wild-type Th2 cells, stochastic monoallelic expression of *Il4* would predict a subpopulation of cells expressing both alleles and consequently twice as much as the cells expressing only one allele. This was not observed.

Here, we show that reexpression of *Il4* by Th2 lymphocytes is not only due to stochastic variations but is determined by activation of NFATc2 by calcineurin. NFAT has 23 phosphorylation sites, which are dephosphorylated by calcineurin in a coop-

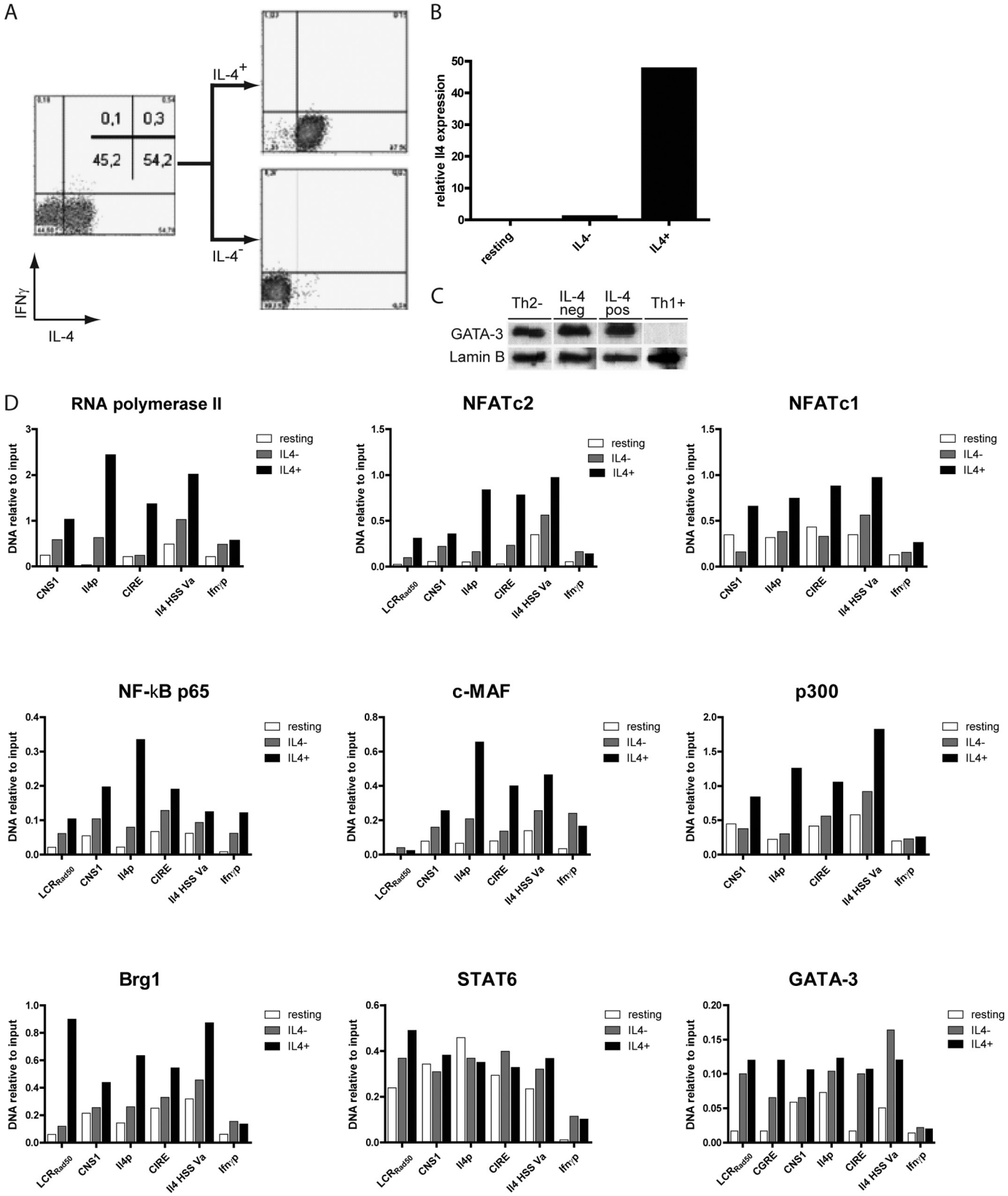
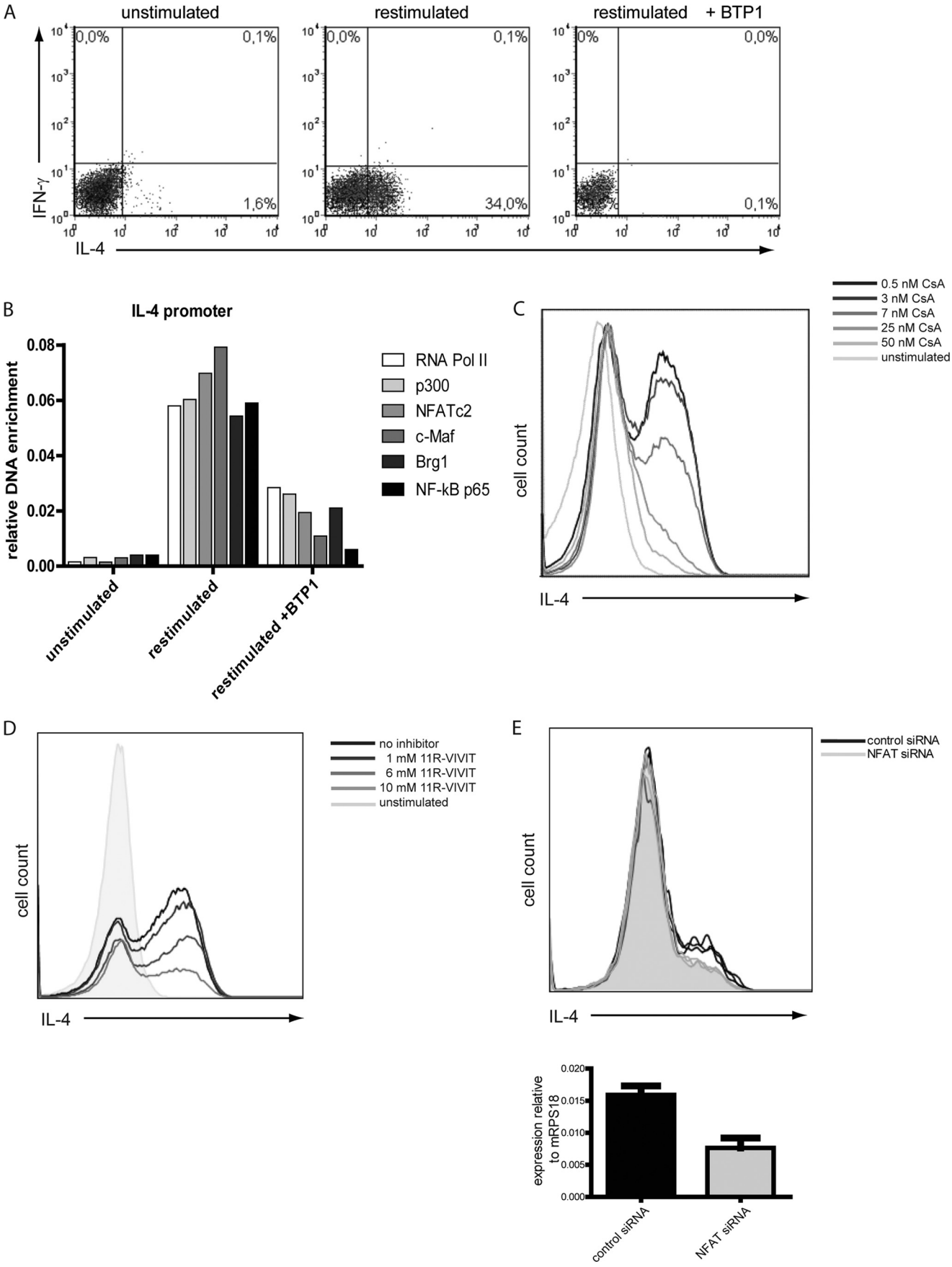


FIGURE 3. **The assembly of a transcription factor complex occurs only in Il-4 expressing Th2 cells.** *A*, Th2 cells polarized for 12 days were restimulated and sorted for IL-4 expression using the IL-4 cytokine secretion assay. *B*, Th2 cells isolated according to IL-4 secretion were lysed, and the *Il4* mRNA expression was quantified in resting Th2 cells, Th2 cells not secreting IL-4, and Th2 cells secreting IL-4. *C*, Th2 cells isolated according to IL-4 secretion were lysed, and the GATA-3 protein expression was quantified by Western blot in resting Th2 cells, Th2 cells not secreting IL-4, Th2 cells secreting IL-4, and restimulated Th1 cells. *D*, Th2 cells polarized for 12 days were either fixed in a resting state or following restimulation and separation into IL-4 secreting and IL-4 non-secreting Th2 cells. ChIP was performed against the indicated transcription factors and probed for binding to regulatory regions in the *Il4* locus. Binding to the *Ifnγ* promoter was used as negative control. Shown are the representative results of three independent experiments.



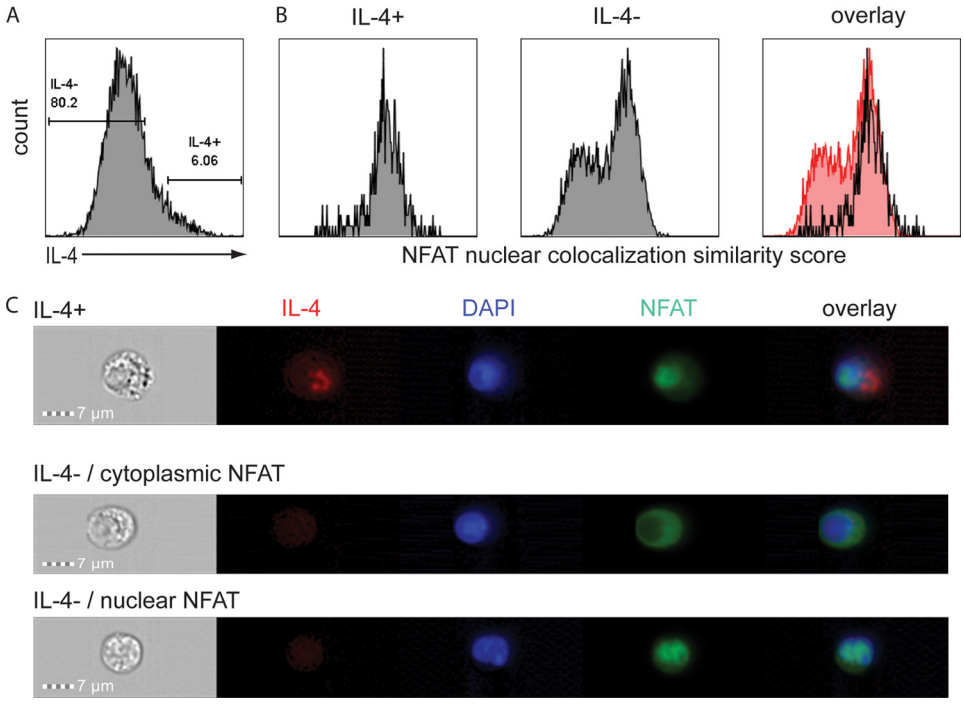


FIGURE 5. **NFATc2 translocates to the nucleus in IL-4 expressing Th2 cells.** *A*, Th2 cells polarized for 12 days were restimulated for 3 h in the presence of 7 nM CsA and stained intracellularly for IL-4 and NFATc2. The cells were analyzed with the image cytometer. Regions indicate IL-4-expressing and non-expressing Th2 cells. *B*, NFATc2 staining was correlated with nuclear DAPI staining in IL-4-expressing and non-expressing Th2 cells. A high similarity score indicates colocalization of NFATc2 with DAPI. *C*, exemplary images of Th2 cells stained for IL-4, NFATc2, and DAPI. Brightfield image, single stain images, and overlay of all stains for all combinations observed are shown.

erative fashion, *i.e.* with strictly sigmoid kinetics, resulting in a "molecular switch" (30). NFAT has to be dephosphorylated at 13 of these sites to expose its nuclear translocation sequence. Dephosphorylation at serine residues at the N-terminal transactivation domain is required for NFAT to bind to its target DNA sequence (18). Translocation of NFATc2 into the nucleus of activated T lymphocytes, thus, is an all-or-none event (Fig. 5*B*) (17). For human Th lymphocytes, this results in all-or-none reexpression of IL-2, which is dependent on TCR signaling strength and mediated by calcineurin (17). Calcineurin and NFATc2, thus, qualify as molecular analog-to-digital converters, translating TCR signaling strength into different frequencies of cells expressing NFAT-dependent genes. In established Th effector/memory cells, it is the epigenetic imprint of a cell determining which genes are accessible, as we show here for murine Th2 lymphocytes.

Interestingly, independent of the frequency of IL-4-expressing cells in a given restimulation, the average amount of IL-4 expressed by the individual Th2 cell is the same, with stochastic cell-to-cell variability (31). This shows that under the conditions analyzed, none of the transcription factors required for *Il4* expression is rate-limiting, except NFAT, as is evident from selective inhibition by BTP1 (32), 11R-VIVIT (22), or siRNA. NFAT is required to assemble GATA-3, RNA polymerase II, NFATc2, NFATc1, NF-κB p65, c-Maf, CBP/p300, Brg1, and STAT6 at the regulatory regions of the *Il4* gene. The transcriptional activator complex may contain more proteins, which have not been analyzed here. STAT6 and GATA-3 did bind to the *Il4* gene also in the absence of NFAT in restimulated Th2 cells not expressing IL-4, and GATA-3 remained bound to the *Il4* gene in Th2 cells expressing IL-4 at late time points of restimulation. Apparently, on their own, they are not competent to assemble any of the other transcription factors analyzed to the *Il4* gene, in particular not CBP/p300 and Brg1, which have been connected to epigenetic imprinting (12, 33). Although GATA-3 itself has been shown to be critical for epigenetic imprinting of the *Il4* gene (26) and is the lineage-determining transcription factor of Th2 cells (5, 25), it is not required for the maintenance of the Th2 phenotype, with respect to IL-4 expression. Unlike inhibition of NFATc2 activity as shown here, conditional deletion of GATA-3 in already established Th2 cells did not change the frequency of Th2 cells reexpressing IL-4 but instead reduced the amount of IL-4 expressed per cell (6). In the Th2 cells analyzed here, GATA-3 expression obviously was not rate-limiting, as both Th2 cells, expressing IL-4 or not, expressed similar amounts of GATA-3.

FIGURE 4. **Calcineurin activity digitalizes IL-4 expression in restimulated Th2 cells.** *A*, Th2 cells polarized for 12 days were restimulated for 3 h in the presence or absence of BTP1, selectively inhibiting the dephosphorylation of NFAT. The expression of IL-4 and IFNγ was determined by intracellular cytokine staining. *B*, Th2 cells restimulated for 3 h in the presence or absence of BTP1 were fixed. ChIP was performed against the indicated transcription factors and probed for binding to the *Il4* promoter. Values are representative of two independent experiments. *C* and *D*, Th2 cells were restimulated for 3 h in the presence of different concentrations of the calcineurin inhibitor CsA or the peptide inhibitor 11R-VIVIT and then stained intracellularly for IL-4 expression. Shown is an overlay of the histogram representation of the IL-4 staining. Shown are the representative results of six and three independent experiments, respectively. *E*, Th2 cells polarized for 6 days and restimulated with anti-CD3 and anti-CD28 antibodies. After 48 h, the cells were treated with NFATc2-specific and control siRNA. Four days later, the cells were restimulated with PMA/ionomycin for 3 h. mRNA was isolated to quantify NFATc2 knockdown efficiency, and expression of IL-4 was analyzed by intracellular staining. Results are representative of three independent experiments. *RNA Pol II*, RNA polymerase II.

The conversion of graded, analog differences in antigen receptor signal strength into expression of defined packages of cytokines in activated T lymphocytes by the calcineurin/NFAT switch, teaches us that in immune reactions, communication between individual cells via NFAT-dependent cytokines occurs in an all-or-none fashion, probably by direct contact and contact-directed secretion (34). This phenomenon is analogous to the signal transduction in neurons, where a stimulus leads to the opening of ion channels and the firing of an action potential. Increasing the strength of the stimulus does not increase the size of the action potential but rather increases the frequency of action potentials (35). This all-or-none principle ensures that neural signals are passed on in full strength once a certain threshold is passed.

Our data indicate that in adapting the magnitude of the immune response to different concentrations of antigens, it is the frequencies of responding cells among those able to respond, which is regulated by the calcineurin/NFAT switch. The advantage of this analog-to-digital conversion would be that the immune system is able to mount immune responses, even if by only a few cells, to antigens of low abundance, and it defines a threshold of reaction for the individual cell, minimizing background expression of potentially harmful genes, *i.e.* immunopathology.

REFERENCES

1. Zhu, J., Yamane, H., and Paul, W. E. (2010) Differentiation of effector CD4 T cell populations (*). *Annu. Rev. Immunol.* **28,** 445–489
2. Asnagli, H., Afkarian, M., and Murphy, K. M. (2002) Cutting edge: Identification of an alternative GATA-3 promoter directing tissue-specific gene expression in mouse and human. *J. Immunol.* **168,** 4268–4271
3. Scheinman, E. J., and Avni, O. (2009) Transcriptional regulation of GATA3 in T helper cells by the integrated activities of transcription factors downstream of the interleukin-4 receptor and T cell receptor. *J. Biol. Chem.* **284,** 3037–3048
4. Höfer, T., Nathansen, H., Löhning, M., Radbruch, A., and Heinrich, R. (2002) GATA-3 transcriptional imprinting in Th2 lymphocytes: a mathematical model. *Proc. Natl. Acad. Sci. U.S.A.* **99,** 9364–9368
5. Ouyang, W., Löhning, M., Gao, Z., Assenmacher, M., Ranganath, S., Radbruch, A., and Murphy, K. M. (2000) Stat6-independent GATA-3 autoactivation directs IL-4-independent Th2 development and commitment. *Immunity* **12,** 27–37
6. Zhu, J., Min, B., Hu-Li, J., Watson, C. J., Grinberg, A., Wang, Q., Killeen, N., Urban, J. F., Jr., Guo, L., and Paul, W. E. (2004) Conditional deletion of Gata3 shows its essential function in T(H)1-T(H)2 responses. *Nat. Immunol.* **5,** 1157–1165
7. Tykocinski, L. O., Hajkova, P., Chang, H. D., Stamm, T., Sözeri, O., Löhning, M., Hu-Li, J., Niesner, U., Kreher, S., Friedrich, B., Pannetier, C., Grütz, G., Walter, J., Paul, W. E., and Radbruch, A. (2005) A critical control element for interleukin-4 memory expression in T helper lymphocytes. *J. Biol. Chem.* **280,** 28177–28185
8. Hutchins, A. S., Mullen, A. C., Lee, H. W., Sykes, K. J., High, F. A., Hendrich, B. D., Bird, A. P., and Reiner, S. L. (2002) Gene silencing quantitatively controls the function of a developmental trans-activator. *Mol. Cell* **10,** 81–91
9. Agarwal, S., Avni, O., and Rao, A. (2000) Cell-type-restricted binding of the transcription factor NFAT to a distal IL-4 enhancer *in vivo*. *Immunity* **12,** 643–652
10. Lee, G. R., Fields, P. E., Griffin, T. J., and Flavell, R. A. (2003) Regulation of the Th2 cytokine locus by a locus control region. *Immunity* **19,** 145–153
11. Lee, D. U., and Rao, A. (2004) Molecular analysis of a locus control region in the T helper 2 cytokine gene cluster: a target for STAT6 but not GATA3. *Proc. Natl. Acad. Sci. U.S.A.* **101,** 16010–16015
12. Wurster, A. L., and Pazin, M. J. (2008) BRG1-mediated chromatin remodeling regulates differentiation and gene expression of T helper cells. *Mol. Cell Biol.* **28,** 7274–7285
13. Hosokawa, H., Tanaka, T., Suzuki, Y., Iwamura, C., Ohkubo, S., Endoh, K., Kato, M., Endo, Y., Onodera, A., Tumes, D. J., Kanai, A., Sugano, S., and Nakayama, T. (2013) Functionally distinct Gata3/Chd4 complexes coordinately establish T helper 2 (Th2) cell identity. *Proc. Natl. Acad. Sci. U.S.A.* **110,** 4691–4696
14. Löhning, M., Richter, A., Stamm, T., Hu-Li, J., Assenmacher, M., Paul, W. E., and Radbruch, A. (2003) Establishment of memory for IL-10 expression in developing T helper 2 cells requires repetitive IL-4 costimulation and does not impair proliferation. *Proc. Natl. Acad. Sci. U.S.A.* **100,** 12307–12312
15. Hu-Li, J., Pannetier, C., Guo, L., Löhning, M., Gu, H., Watson, C., Assenmacher, M., Radbruch, A., and Paul, W. E. (2001) Regulation of expression of IL-4 alleles: analysis using a chimeric GFP/IL-4 gene. *Immunity* **14,** 1–11
16. Guo, L., Hu-Li, J., and Paul, W. E. (2005) Probabilistic regulation in TH2 cells accounts for monoallelic expression of IL-4 and IL-13. *Immunity* **23,** 89–99
17. Podtschaske, M., Benary, U., Zwinger, S., Höfer, T., Radbruch, A., and Baumgrass, R. (2007) Digital NFATc2 activation per cell transforms graded T cell receptor activation into an all-or-none IL-2 expression. *PLoS One* **2,** e935
18. Okamura, H., Aramburu, J., García-Rodríguez, C., Viola, J. P., Raghavan, A., Tahiliani, M., Zhang, X., Qin, J., Hogan, P. G., and Rao, A. (2000) Concerted dephosphorylation of the transcription factor NFAT1 induces a conformational switch that regulates transcriptional activity. *Mol. Cell* **6,** 539–550
19. Chang, H. D., Helbig, C., Tykocinski, L., Kreher, S., Koeck, J., Niesner, U., and Radbruch, A. (2007) Expression of IL-10 in Th memory lymphocytes is conditional on IL-12 or IL-4, unless the IL-10 gene is imprinted by GATA-3. *Eur. J. Immunol.* **37,** 807–817
20. Stittrich, A. B., Haftmann, C., Sgouroudis, E., Kühl, A. A., Hegazy, A. N., Panse, I., Riedel, R., Flossdorf, M., Dong, J., Fuhrmann, F., Heinz, G. A., Fang, Z., Li, N., Bissels, U., Hatam, F., Jahn, A., Hammoud, B., Matz, M., Schulze, F. M., Baumgrass, R., Bosio, A., Mollenkopf, H. J., Grün, J., Thiel, A., Chen, W., Höfer, T., Loddenkemper, C., Löhning, M., Chang, H. D., Rajewsky, N., Radbruch, A., and Mashreghi, M. F. (2010) The microRNA miR-182 is induced by IL-2 and promotes clonal expansion of activated helper T lymphocytes. *Nat. Immunol.* **11,** 1057–1062
21. Trevillyan, J. M., Chiou, X. G., Chen, Y. W., Ballaron, S. J., Sheets, M. P., Smith, M. L., Wiedeman, P. E., Warrior, U., Wilkins, J., Gubbins, E. J., Gagne, G. D., Fagerland, J., Carter, G. W., Luly, J. R., Mollison, K. W., and Djuric, S. W. (2001) Potent inhibition of NFAT activation and T cell cytokine production by novel low molecular weight pyrazole compounds. *J. Biol. Chem.* **276,** 48118–48126
22. Frischbutter, S., Gabriel, C., Bendfeldt, H., Radbruch, A., and Baumgrass, R. (2011) Dephosphorylation of Bcl-10 by calcineurin is essential for canonical NF-κB activation in Th cells. *Eur. J. Immunol.* **41,** 2349–2357
23. Aramburu, J., Yaffe, M. B., López-Rodríguez, C., Cantley, L. C., Hogan, P. G., and Rao, A. (1999) Affinity-driven peptide selection of an NFAT inhibitor more selective than cyclosporin A. *Science* **285,** 2129–2133
24. Wei, G., Wei, L., Zhu, J., Zang, C., Hu-Li, J., Yao, Z., Cui, K., Kanno, Y., Roh, T. Y., Watford, W. T., Schones, D. E., Peng, W., Sun, H. W., Paul, W. E., O'Shea, J. J., and Zhao, K. (2009) Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in lineage fate determination of differentiating CD4+ T cells. *Immunity* **30,** 155–167
25. Zheng, W., and Flavell, R. A. (1997) The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* **89,** 587–596
26. Lee, G. R., Fields, P. E., and Flavell, R. A. (2001) Regulation of IL-4 gene expression by distal regulatory elements and GATA-3 at the chromatin level. *Immunity* **14,** 447–459
27. Lee, D. U., Agarwal, S., and Rao, A. (2002) Th2 lineage commitment and efficient IL-4 production involves extended demethylation of the IL-4 gene. *Immunity* **16,** 649–660
28. Guo, L., Hu-Li, J., and Paul, W. E. (2004) Probabilistic regulation of IL-4 production in Th2 cells: accessibility at the Il4 locus. *Immunity* **20,**

193–203
29. Rivière, I., Sunshine, M. J., and Littman, D. R. (1998) Regulation of IL-4 expression by activation of individual alleles. *Immunity* **9,** 217–228
30. Salazar, C., and Höfer, T. (2005) Activation of the transcription factor NFAT1: concerted or modular regulation? *FEBS Lett.* **579,** 621–626
31. Mariani, L., Schulz, E. G., Lexberg, M. H., Helmstetter, C., Radbruch, A., Löhning, M., and Höfer, T. (2010) Short-term memory in gene induction reveals the regulatory principle behind stochastic IL-4 expression. *Mol. Syst. Biol.* **6,** 359
32. Frischbutter, S., Schultheis, K., Pätzel, M., Radbruch, A., and Baumgrass, R. (2012) Evaluation of calcineurin/NFAT inhibitor selectivity in primary human Th cells using bar-coding and phospho-flow cytometry. *Cytometry A* **81,** 1005–1011
33. Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H., and Nakatani, Y. (1996) The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell* **87,** 953–959
34. Kupfer, A., Mosmann, T. R., and Kupfer, H. (1991) Polarized expression of cytokines in cell conjugates of helper T cells and splenic B cells. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 775–779
35. Walmsley, B., Alvarez, F. J., and Fyffe, R. E. (1998) Diversity of structure and function at mammalian central synapses. *Trends Neurosci.* **21,** 81–88

European Journal of Immunology

Technical comment

[DOI: 10.1002/eji.201445247]

A Ca^{2+} concentration of 1.5 mM, as present in IMDM but not in RPMI, is critical for maximal response of Th cells to PMA/ionomycin

In the original activation of T lymphocytes, distinct cytokine genes and genes for other functionally relevant proteins are imprinted for reexpression upon secondary activation by antigen [1, 2]. To measure this imprinting independently of a particular antigen, T cells can be stimulated with PMA and ionomycin, mimicking TCR stimulation [3]. PMA directly activates protein kinase C and hence leads to the nuclear translocation of NF-κB via phosphorylation of its antagonist IκB [4–6]. Ionomycin induces increased cytoplasmic Ca^{2+} concentrations that activate calcineurin [7, 8]. Calcineurin dephosphorylates NFAT and thus enables its translocation to the nucleus [9–11]. NFAT, NF-κB, and AP-1, which are also activated by PMA/ionomycin, induce the transcription of imprinted genes [1, 12, 13]. The nuclear translocation of NFAT occurs in an all-or-none fashion, due to the multiple dephosphorylation events required, a process mediated by the Ca^{2+}-dependent phosphatase calcineurin [11]. Thus, when a population of T cells is restimulated, calcineurin activity determines the frequency of cytokine-producing cells in that population, rather than the amount of cytokine expressed per cell [14, 15].

When addressing the functional potential of Th cells in murine T cell transfer-induced colitis [16], we compared the frequencies of cytokine-producing mucosal Th cells, isolated ex vivo and restimulated with PMA and ionomycin in RPMI (Roswell Park Memorial Institute 1640) or in IMDM (Iscove's modified Dulbecco's medium). In IMDM, the frequencies of Th cells expressing IFN-γ, IL-17, IL-10, IL-22, or TNF were consistently higher than in RPMI. The difference in frequencies ranged from 1.4-fold for IFN-γ to more than threefold for TNF (Fig. 1 and Supporting Information Fig. 1). This effect was also observed for Th cells from spleen and mesenteric LNs of colitic mice (data not shown).

One major difference in the formulations of RPMI versus IMDM of potential relevance for PMA/ionomycin stimulation is the concentration of Ca^{2+}. While RPMI contains 0.42 mM Ca^{2+}, IMDM contains 1.49 mM. Indeed, supplementation of RPMI with Ca^{2+} to a total concentration of 1.5 mM was sufficient to trigger frequencies of cytokine-producing cells in RPMI comparable to IMDM (Fig. 1). Increasing the Ca^{2+} concentration of RPMI even further, from 1.5 to 2.5 and 3.5 mM, had no significant effect on the frequencies of IL-17- or IL-22-expressing Th cells (Fig. 2A). For IFN-γ, we observed a minor increase upon restimulation with 2.5 mM of Ca^{2+} compared with that at 1.5 mM (Fig. 2A, middle). Taken together, a Ca^{2+} concentration of the medium of at least 1.5 mM reveals the maximal frequencies of cytokine-expressing Th cells upon PMA/ionomycin stimulation.

Conversely, lowering the concentration of available Ca^{2+} of IMDM by the Ca/Mg-chelating reagent EDTA, resulted in a dose-dependent reduction in the frequencies of cytokine-expressing cells (Fig. 2B). Since EDTA binds Ca^{2+} in an equimolar fashion and with 100× higher affinity than Mg^{2+} [17], 1 mM of EDTA reduced the concentration of available Ca^{2+} from 1.49 mM to approximately 0.5 mM, comparable to the Ca^{2+} concentration of RPMI ($[Ca^{2+}] = 0.42$ mM). Indeed, addition of 1.0 mM EDTA to IMDM reduced the frequencies of cytokine-producing cells to those obtained in RPMI, as shown for IL-17, IFN-γ, and IL-22 in Figure 2B.

Maximizing the PMA/ionomycin stimulation by Ca^{2+} supplementation of RPMI had minimal impact on the viability of the activated Th cells, nor did it change the kinetics of cytokine expression. The viability of the Th cells 4 h after onset of PMA/ionomycin treatment was 78.6 ± 1.8 in RPMI, 76.7 ± 1.8 in IMDM, and 77.3 ± 2.1 in $CaCl_2$-supplemented RPMI (data not shown). The frequencies of cytokine-expressing Th cells differed between media, but were comparable at 4, 6, and 8 h after onset of each stimulation, as shown for IL-17-, IFN-γ-, and IL-22-expressing Th cells in Figure 2C. In particular, the frequencies of cytokine-producing Th cells in RPMI did not increase at 6 or 8 h to frequencies in IMDM or in $CaCl_2$-supplemented RPMI (Fig. 2C).

We show here that conventional RPMI, routinely used for the activation of Th lymphocytes with PMA and ionomycin, contains too little Ca^{2+} for maximal ionomycin stimulation. A significant underestimation

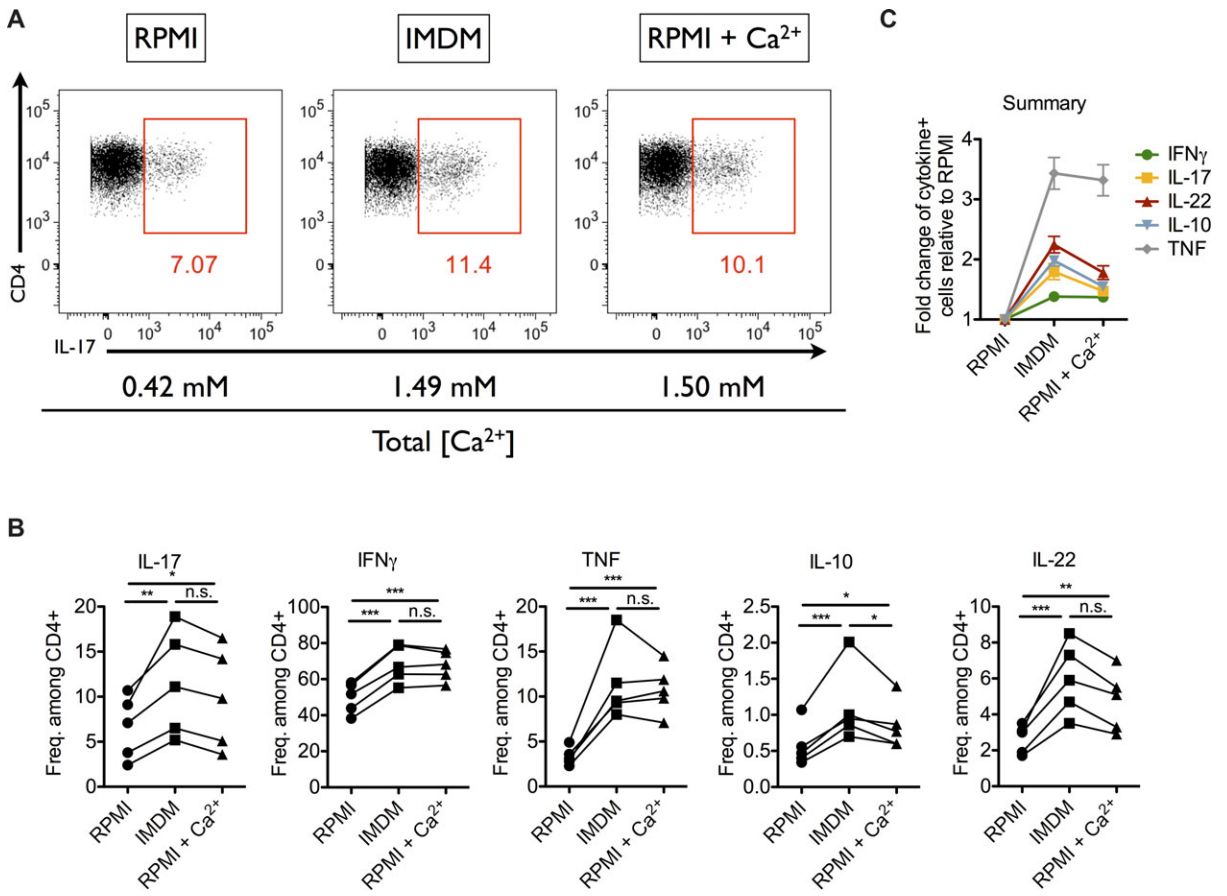


Figure 1. Frequencies of cytokine-expressing Th cells upon restimulation with PMA/ionomycin in RPMI, IMDM, and Ca^{2+}-supplemented RPMI. Small intestine lamina propria (SI LP) cells of colitic mice were stimulated with PMA/ionomycin in RPMI, IMDM, or Ca^{2+}-supplemented RPMI. (A) Intracellular staining for IL-17 in SI LP Th cells. (B) Frequencies of IL-17, IFN-γ, TNF, IL-10, and IL-22-expressing cells among SI LP Th cells stimulated in RPMI, IMDM, and Ca^{2+}-supplemented RPMI, as determined by flow cytometry. Lines represent cells from individual mice. (C) Fold change in frequencies of cytokine-expressing cells, stimulated in IMDM or Ca^{2+}-supplemented RPMI compared to RPMI, as determined by flow cytometry. Data are shown as mean ± SEM, $n = 5$ mice/group and are from one experiment representative of four independent experiments with similar results. (B) $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ by one-way ANOVA for repeated measurements and Tukey's post hoc test.

of antigen-experienced Th cells imprinted for expression of distinct cytokines is the consequence, as demonstrated here for Th cells isolated ex vivo from inflamed tissue or secondary lymphoid organs of mice with colitis. Increasing the Ca^{2+} concentration of RPMI from the regular 0.42 to ≥1.5 mM is sufficient to correct this effect and obtain a more accurate estimation of the functional potential of polyclonal Th cell populations.

Methods

Mice

Specific pathogen free C57BL/6J and C57BL/6 *Rag1*$^{-/-}$ mice were obtained from Charles River (Sulzfeld, Germany). All animal experiments were performed in accordance with institutional, state, and federal guidelines.

T cell transfer colitis

Colitis was induced as described before [16]. Briefly, $CD4^{+}$ T cells from spleen and LNs of C57BL/6J donors were purified by MACS using mouse CD4 microbeads (L3T4, Miltenyi Biotec). Viable $CD4^{+}CD45RB^{hi}CD25^{-}$ cells were isolated by FACS. A total of 4×10^{5} cells were injected i.v. into each of the C57BL/6 *Rag1*$^{-/-}$ recipients. Mice were sacrificed 2–3 weeks after transfer, when signs of diarrhea and weight loss became apparent.

Isolation of lamina propria (LP) mononuclear cells

LP mononuclear cells were isolated from colon and small intestine (SI) as described before [18]. In brief, fat was removed from colon and SI. They were then opened longitudinally and washed with PBS. The epithelial layer was stripped off by two rounds of incubation in calcium/magnesium-free HBSS with 5 mM EDTA and 10 mM HEPES for 20 min at 37°C with 100 RPM shaking. To obtain a single cell suspension of the LP, colons and SIs were minced into small pieces and incubated three times for 20 min with 0.5 mg/mL Collagenase D (Roche), 0.5 mg/mL DNase I (Sigma), and 0.05 U/mL Dispase (BD)

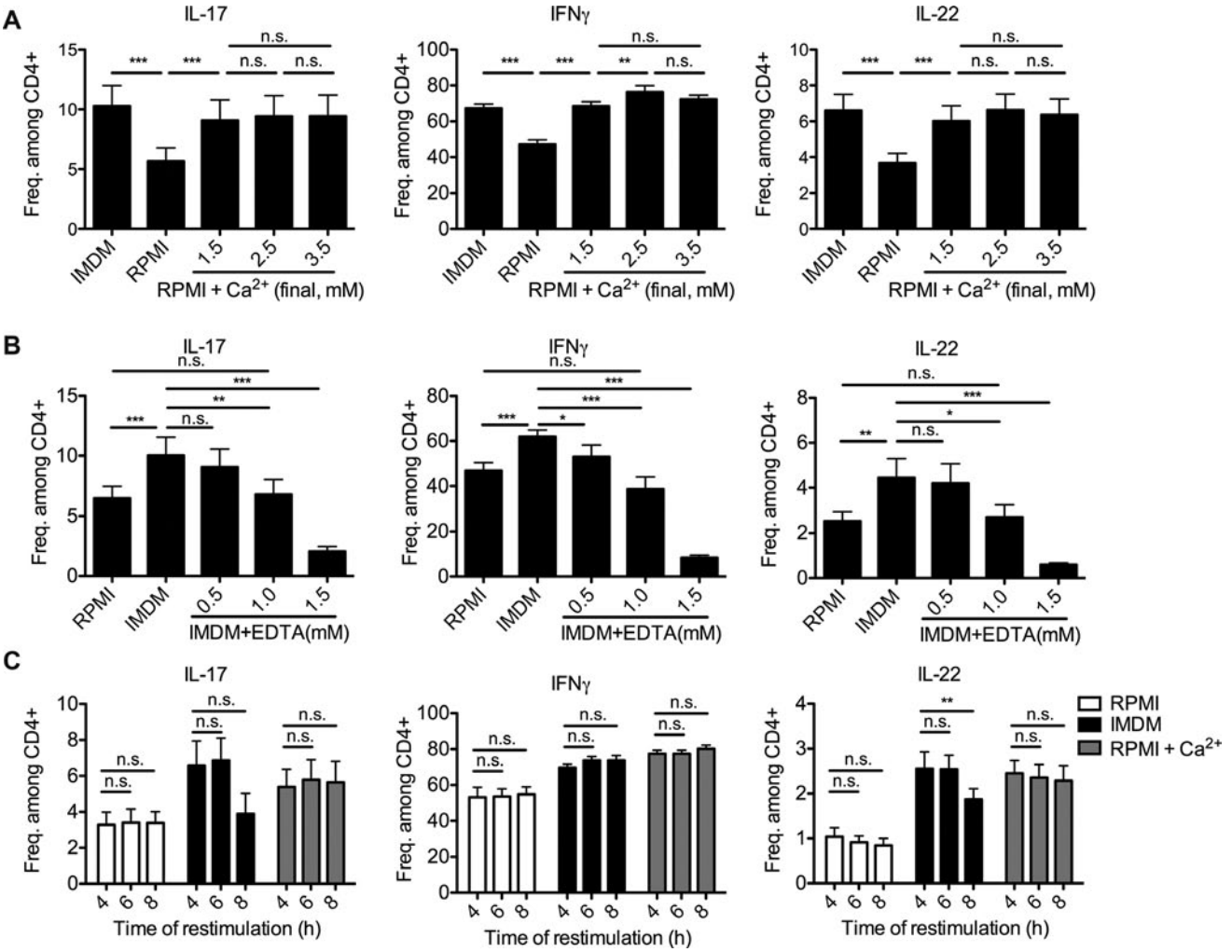


Figure 2. Quantitative and kinetic analysis of cytokine expression of Th cells restimulated with PMA/ionomycin in different Ca^{2+} concentrations. (A, B) SI LP cells of colitic mice were stimulated with PMA/ionomycin in IMDM, RPMI, or (A) Ca^{2+}-supplemented RPMI or (B) IMDM supplemented with EDTA. (A) The frequencies of Th cells expressing IL-17, IFN-γ, and IL-22 were measured by flow cytometry. Data are shown as mean + SEM of 9 mice/group, pooled from two independent experiments. (B) The frequencies of Th cells expressing IL-17, IFN-γ, and IL-22 were measured by flow cytometry. Data are shown as mean + SEM of 9 mice/group, pooled from two independent experiments. (C) LP Th cells of colitic mice were stimulated in RPMI, IMDM, or Ca^{2+}-supplemented RPMI for 4, 6, or 8 h. The frequencies of Th cells expressing IL-17, IFN-γ, and IL-22 were measured by flow cytometry. Data are shown as mean + SEM of 4 mice/group, from one experiment. (A–C) $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ by one-way ANOVA for repeated measurements and Tukey's post hoc test.

in calcium/magnesium-containing HBSS with 10 mM HEPES at 37°C with 100 RPM shaking. For the SI, LP mononuclear cells were separated from debris by centrifugation over a Percoll gradient (GE Healthcare).

Restimulation and flow cytometry

Ca^{2+}-supplemented RPMI was obtained by adding 1.08 mM of $CaCl_2$ to RPMI ($[Ca^{2+}_{final}] = 1.5$ mM). IMDM ($[Ca^{2+}] = 1.49$ mM), RPMI (Life Technologies, $[Ca^{2+}] = 0.42$ mM), and Ca^{2+}-supplemented RPMI ($[Ca^{2+}] = 1.50$ mM) were supplemented with 10% FCS and contained 25 mM HEPES. For intracellular cytokine staining, 1–2 × 10^6 cells were restimulated with 10 ng/mL PMA (Sigma) and 1 μg/mL ionomycin (Santa Cruz) in the respective media at 5 × 10^6–1 × 10^7 cells/mL for a total of 4 h. After 1 h, brefeldin A (BioLegend) was added to a final concentration of 5 μg/mL. Following washing with PBS, cells were stained with a fixable live/dead staining (pacific orange succinimidyl ester, Life Technologies) for 20 min on ice. After 20 min fixation using the BD Cytofix/Cytoperm buffer, cells were washed with and stained in 0.5% w/v Saponin (Sigma) for 20 min on ice. A total of 1–2 × 10^5 cells were measured with a FACSCanto II (BD).

Antibodies

Epitope	Color	Clone	Manufacturer
CD3	Allophycocyanin-eFluor 780	145–2C11	eBioscience
CD4	Pe-Cy7	RM4–5	eBioscience
IFN-γ	PerCP-Cy5.5	XMG1.2	eBioscience
IL-17	FITC	TC11–18H10	BioLegend
IL-10	Pe	JES5–16e3	eBioscience
IL-22	Allophycocyanin	IL22JOP	eBioscience
TNF	AlexaFluor405	MP6-XT22	Custom

Data presentation and statistics

Individual data points refer to Th cells isolated from individual colitic mice and are presented as mean + SEM unless stated otherwise. Multiple comparisons were tested for significant differences by one-way ANOVA for repeated measurements followed by Tukey's post hoc test with $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Jakob Zimmermann, Andreas Radbruch and Hyun-Dong Chang

Cell Biology Group, Deutsches Rheumaforschungszentrum, Berlin, Germany, a Leibniz Institute

Acknowledgments: We are grateful to P. Saikali for fruitful discussions. We would like to thank C. Winsauer, A. Okhrimenko, M. Bardua, C. Haftmann, K. Lehmann, P. Maschmeyer, R. Riedel, F. Siracusa, M. Weber, and K. Westendorf for the provision of samples and/or experimental support. We are grateful to J. Kirsch and T. Kaiser, operators of the flow cytometry core facility (FCCF). This work was supported by the German Research Council (DFG SFB633, SFB 650 and GRK1121) and the European Research Council advanced grant ERC-2010-AdG_20100317 Grant 268987. J.Z. designed and performed the research, analyzed the data, and wrote the paper. A.R. designed and supervised the research and wrote the paper. H.-D. C. designed the research, analyzed the data, and wrote the paper.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

References

1 **Löhning, M. et al.**, *Adv Immunol.* 2002. **80**: 115–181.

2 **Rebhahn, J. A. et al.**, *Eur. J. Immunol.* 2014. **44**: 2216–2229.

3 **Truneh, A. et al.**, *Nature* 1985. **313**: 318–320.

4 **Castagna, M. et al.**, *J. Biol. Chem.* 1982. **257**: 7847–7851.

5 **Sen, R. and Baltimore, D.**, *Cell* 1986. **47**: 921–928.

6 **Ghosh, S. and Baltimore, D.**, *Nature* 1990. **344**: 678–682.

7 **Lyall, R. M. et al.**, *Biochem. Soc. Trans.* 1980. **8**: 720–721.

8 **Yang, S. D. et al.**, *Biochem. Biophys. Res. Commun.* 1982. **106**: 1419–1425.

9 **Jain, J. et al.**, *Nature* 1993. **365**: 352–355.

10 **Shaw, K. T. et al.**, *Proc. Natl. Acad. Sci. USA* 1995. **92**: 11205–11209.

11 **Okamura, H. et al.**, *Mol. Cell.* 2000. **6**: 539–550.

12 **Su, B. et al.**, *Cell* 1994. **77**: 727–736.

13 **Rao, A. et al.**, *Annu. Rev. Immunol.* 1997. **15**: 707–747.

14 **Koeck, J. et al.**, *J. Biol. Chem.* 2014. **289**: 26752–26761.

15 **Podtschaske, M. et al.**, *PLoS One* 2007. **2**: e935.

16 **Powrie, F. et al.**, *Int. Immunol.* 1993. **5**:1461–1471.

17 **Skoog, D. A., West, D. M. and Holler, F. J.**, *Fundamentals of analytical chemistry*. Saunders College Publishing, New York 1988, p. 418.

18 **Sanos, S. L. and Diefenbach, A.**, *Methods Mol. Biol.* 2010. **612**: 505–517.

Abbreviations: **IMDM**: Iscove's modified Dulbecco's medium · **LP**: lamina propria · **RPMI**: Roswell Park Memorial Institute 1640 · **SI**: small intestine

Keywords: CD4+ T cells • Cellular activation • Cytokines • Flow cytometry • PMA/ionomycin

Full correspondence: Dr. Hyun-Dong Chang, Deutsches Rheumaforschungszentrum Berlin, Charitéplatz 1, 10117 Berlin, Germany
Fax: +49-(0)30-28460-603
e-mail: chang@drfz.de

Received: 8/10/2014
Revised: 21/11/2014
Accepted: 22/12/2014
Accepted article online: 26/12/2014

The detailed *Materials and methods* for Technical comments are available online in the Supporting information

3.4 Das IFNG-Gen ist epigenetisch geprägt, IL10 nicht

Dong J, Ivascu C, **Chang HD**, Wu P, Angeli R, Maggi L, Eckhardt F, Tykocinski L, Haefliger C, Möwes B, Sieper J, Radbruch A, Annunziato F, Thiel A. 2007. IL-10 is excluded from the functional cytokine memory of human CD4+ memory T lymphocytes. *J Immunol.* 179(4):2389-96.

Dong J, **Chang HD**, Ivascu C, Qian Y, Rezai S, Okhrimenko A, Cosmi L, Maggi L, Eckhardt F, Wu P, Sieper J, Alexander T, Annunziato F, Gossen M, Li J, Radbruch A, Thiel A. 2013. Loss of methylation at the IFNG promoter and CNS-1 is associated with the development of functional IFN-γ memory in human CD4(+) T lymphocytes. *Eur J Immunol.* 43(3):793-804.

Th-Zellen zeichnen sich durch ein Gedächtnis für Zytokinexpression aus. Dieses Gedächtnis basiert sowohl auf der epigenetischen Prägung der entsprechenden Zytokingene als auch der stabilen Expression von entscheidenden Transkriptionsfaktoren, wie T-bet für IFNγ und RORγt für IL-17. In Studien an Maus Th-Zellen konnte gezeigt werden, dass die IFNγ-Expression in Th1-Zellen auch unter z.B. Th2-polarisierenden Bedingungen sehr stabil ist, während IL-10 in Abwesenheit von instruierenden Signalen nicht reexprimiert wird. Die molekulare Grundlage für den Unterschied im Zytokingedächtnis für IFNγ und IL-10 ist unklar.
Wir haben die epigenetische Prägung von *IFNG* und *IL10* in humanen Gedächtnis-Th-Zellen untersucht. Hierzu haben wir Th-Zellen aus peripherem Blut von gesunden Spendern anhand ihrer IFNγ- und IL-10-Expression nach Restimulation mittels des Zytokinsekretionsassays in IFNγ-Einzelproduzenten, IL-10-Einzelproduzenten und IFNγ/IL-10-Doppelproduzenten aufgetrennt. Im *IFNG*- und *IL10*-Gen haben wir genomische Regionen mit hoher Sequenzhomologie zwischen Maus und Mensch und hohem CpG-Gehalt als potentielle regulatorische Elemente identifiziert. Die DNA-Methylierungsanalyse dieser potentiellen regulatorischen Regionen hat gezeigt, dass das *IL10*-Gen sich in der DNA-Methylierung nicht zwischen IL-10 exprimierenden oder nicht-exprimierenden Th-Zellen unterscheidet. Demnach scheint das *IL10*-Gen epigenetisch nicht geprägt zu sein. Im Gegensatz dazu war sowohl der Promoter und die CNS-1 (*conserved non-coding sequence* 1) des *IFNG*-Gens in Th-Zellen, die IFNγ exprimierten vollständig demethyliert. Um zu testen, ob die DNA-Methylierung der entsprechenden Zytokingene funktionelle Auswirkungen haben, wurden Th-Zellen, die IFNγ, IL-

10 oder beide Zytokine exprimieren *in vitro* kultiviert und eine Woche später nochmals restimuliert, um die Zytokinreexpression zu testen. Th-Zellen, die IFNγ exprimierten, haben auch in der nachfolgenden Restimulation IFNγ exprimiert, während ehemalige IL-10-Produzenten eine Woche nach Restimulation kein IL-10 mehr exprimierten. Unsere Daten deuten darauf hin, dass Th- Zellen das *IL10* Gen nicht epigenetisch prägen und auch kein funktionelles Gedächtnis für IL-10 ausbilden. *IFNG*, andererseits, wird epigenetisch durch DNA Demethylierung und auch funktionell für Reexpression nach Restimulation geprägt.

IL-10 Is Excluded from the Functional Cytokine Memory of Human CD4$^+$ Memory T Lymphocytes[1]

Jun Dong,[2,3]* Claudia Ivascu,[2‡] Hyun-Dong Chang,[†] Peihua Wu,*[§] Roberta Angeli,[¶] Laura Maggi,[¶] Florian Eckhardt,[‡] Lars Tykocinski,[†] Carolina Haefliger,[‡] Beate Möwes,* Jochen Sieper,[§] Andreas Radbruch,[†] Francesco Annunziato,[¶] and Andreas Thiel[3]*

Epigenetic modifications, including DNA methylation, profoundly influence gene expression of CD4$^+$ Th-specific cells thereby shaping memory Th cell function. We demonstrate here a correlation between a lacking fixed potential of human memory Th cells to re-express the immunoregulatory cytokine gene *IL10* and its DNA methylation status. Memory Th cells secreting IL-10 or IFN-γ were directly isolated ex vivo from peripheral blood of healthy volunteers, and the DNA methylation status of *IL10* and *IFNG* was assessed. Limited difference in methylation was found for the *IL10* gene locus in IL-10-secreting Th cells, as compared with Th cells not secreting IL-10 isolated directly ex vivo or from in vitro-established human Th1 and Th2 clones. In contrast, in IFN-γ^+ memory Th cells the promoter of the *IFNG* gene was hypomethylated, as compared with IFN-γ-nonsecreting memory Th cells. In accordance with the lack of epigenetic memory, almost 90% of ex vivo-isolated IL-10-secreting Th cells lacked a functional memory for IL-10 re-expression after restimulation. Our data indicate that *IL10* does not become epigenetically marked in human memory Th cells unlike effector cytokine genes such as *IFNG*. The exclusion of IL-10, but not effector cytokines, from the functional memory of human CD4$^+$ T lymphocytes ex vivo may reflect the need for appropriate regulation of IL-10 secretion, due to its potent immunoregulatory potential. *The Journal of Immunology,* **2007, 179: 2389–2396.**

Immune reactions are tightly controlled to avoid excessive activation in the course of pathogen-specific immune responses and to suppress the activation of autoreactive lymphocytes. Different subsets of regulatory T cells represent pivotal players of immune regulation in the immune system (1). Populations of regulatory T cells identified include naturally occurring CD4$^+$CD25$^+$ T regulatory cells (2), Ag-induced T regulatory type 1 cells (Tr1)[4] (3), and Th3 cells (4), which all display certain abilities to produce the immunoregulatory cytokine IL-10. IL-10 exerts its inhibitory action on macrophages and dendritic cells, thereby regulating effector cell activation, but has also stimulatory effects on B and T cells (5). *Il10*-deficient mice develop severe chronic enterocolitis (6) while tissue- or cell-specific overexpression of *Il10* leads to impaired immune responses (7–10). Tr1 cells secret high levels of IL-10 and low levels of IFN-γ and IL-2 upon activation, but display their regulatory function in an IL-10-dependent manner (11–14). IL-10 production by CD4$^+$CD25$^+$ regulatory T cells has been reported in different in vivo (15, 16) and in vitro (17) experimental systems. Thus, *IL10* gene expression by regulatory T cell subsets is an essential factor for effective regulation of immune responses.

The immune system needs to act rapidly and specifically to pathogens but also has to control autoimmunity. These actions are partially mediated by effector and regulatory cytokines produced by memory Th cells that memorize the expression of cytokine genes after primary activations triggered by TCR signaling and instructive costimulatory signals, and re-express memorized cytokine genes within a few hours upon restimulation via TCR, in the absence of original instructive costimulatory signals (18). Control of effector cytokines expression via networks of transcription factors and epigenetic regulation has been well documented. Epigenetic modifications such as changes in DNA methylation (primarily at the C5 position of cytosine in CpG dinucleotides), histone modifications, and chromatin rearrangement within the nucleus influence the accessibility of transcription factors to their DNA binding sites. These associated molecular changes are heritable and provide a basis for memory of gene expression (19–22). Indeed, these mechanisms have been documented for Th1 and Th2 cells in the transcriptional regulation of the *Ifng/IFNG* and *Il4* clustered genes (23–30). However, epigenetic regulation of *IL10*, especially by DNA methylation, has remained poorly understood.

The expression of *Il10* is under the control of several transcription factors such as Stat3 (31), Sp1 (32) and Sp3 (33), NF-κB (34), Smad-4 (35), c-Maf (36), and Jun proteins (37). Recent data obtained in mouse on *Il10* gene accessibility according to DNase I hypersensitivity have suggested chromatin remodeling of the *Il10* gene locus. However, some DNase I hypersensitive sites (HSS) were described as both enhancing and silencing *Il10* gene expression (34, 38, 39).

To gain insight into the epigenetic regulation of the *IL10* gene in comparison to the *IFNG* gene in human Th cells, we characterized

*Clinical Immunology Group and [†]Cell Biology Group, Deutsches Rheuma-Forschungszentrum Berlin, Berlin, Germany; [‡]Epigenomics, Berlin, Germany; [§]Department of Rheumatology, Charité Campus Mitte, Berlin, Germany; and [¶]Center of Research, Transfer, High Education MCIDNENT, University of Florence, Firenze, Italy

Received for publication December 12, 2006. Accepted for publication May 16, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by grants from the Deutsche Forschungsgemeinschaft (TH 806/1-1, SFB421 TP B6, SFB 650 TP 11, and BMBF 312102).

[2] J.D. and C.I. contributed equally to this study.

[3] Address correspondence and reprint requests to Dr. Andreas Thiel and Dr. Jun Dong, Clinical Immunology, Deutsches Rheuma-Forschungszentrum Berlin, Charitéplatz 1, Berlin, Germany. E-mail addresses: thiel@drfz.de and dong@drfz.de

[4] Abbreviations used in this paper: Tr1, T regulatory type 1 cell; P/I, PMA/ionomycin; HSS, hypersensitivity site; ChIP, chromatin immunoprecipitation; H3Ac, histone 3 acetylation; H3K4me3, histone 3 lysine 4 trimethylation; ROI, regions of interest; Dnmt, DNA methyltransferase.

IL-10$^+$IFN-γ^-, IL-10$^+$IFN-γ^+, IL-10$^-$IFN-γ^+, and IL-10$^-$IFN-γ^- Th cell subsets isolated directly ex vivo from peripheral blood of healthy adults. Using a bisulfite-modified DNA sequencing approach, we performed semiquantitative assessments of the DNA methylation pattern of the entire *IL10* gene locus spanning 9.1 kb upstream and 9 kb downstream of the transcriptional start site that encompasses 88 selected CpGs. We demonstrate the lack of a specific DNA methylation pattern of the *IL10* gene in IL-10-secreting Th cells isolated from ex vivo- and in vitro-established Ag-specific human Th1 and Th2 clones. However, we show an unambiguous hypomethylation of the *IFNG* gene promoter in different IFN-γ-producing Th cell subsets. Thus, contrary to the effector cytokine gene *IFNG*, expression of the immunoregulatory cytokine gene *IL10* is not mainly regulated by DNA methylation. Moreover, IL-10-producing Th cells lack a memory for IL-10 re-expression in vitro. In conclusion, IL-10 is excluded from the functional cytokine memory in human Th cells, preventing the generation of memory Th cells with an inherited program to secrete IL-10, possibly to ensure a limited effect of IL-10 in down-regulation of adaptive pathogen-specific immunity.

Materials and Methods

Media and reagents

The media used were RPMI 1640 supplemented with 1% glutamax, 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen Life Technologies), and 10% human AB serum (PAA). PMA (P; 5 ng/ml; Sigma-Aldrich) and 1 μg/ml ionomycin (I; Sigma-Aldrich) were used for stimulation. rIL-7 and rIL-15, each 10 μg/ml (R&D Systems), and 5–10 μM 5-azacytidine (Sigma-Aldrich) were applied to in vitro cell cultures. Brefeldin A (5 μg/ml; Sigma-Aldrich) was used to block cytokine secretion. The following Abs were used for FACS analysis: anti-CD69 FITC, anti-IFN-γ PerCP Cy5.5, anti-IL-10 allophycocyanin, anti-CD45RO (allophycocyanin), anti-CD45RA (allophycocyanin), -CCR7 (FITC; BD Biosciences), and anti-CD4 Cy5 (TT1, mouse IgG1, house conjugate).

Sample collection and preparation

Buffy coats from healthy adult anonymous donors were obtained in accordance with local ethical committee approval. PBMCs were isolated from each buffy coat by density gradient sedimentation using Ficoll-Hypaque (Sigma-Aldrich). Cells were washed twice with PBS before CD4$^+$ T cell separation.

Purification, sorting of CD4$^+$ T lymphocytes

CD4$^+$ T cells were purified from PBMCs by MACS using CD4 microbeads (Miltenyi Biotec). The purity of the sorted population was 95–99%, as determined by FACSCalibur using CellQuest software (BD Biosciences).

Isolation of Th cell subsets secreting IL-10 or IFN-γ or both

Human Th1 and Th2 clones, highly purified CD4$^+$ T lymphocytes or CD4$^+$ T lymphocytes labeled with CD45RO or CD45RA and CCR7 were stimulated with P/I, followed by detection and isolation of IL-10-secreting and IFN-γ- and/or IL-10-secreting Th cell subsets using single or double cytometric cytokine secretion assays (Miltenyi Biotec) and a FACSDiva (BD Biosciences). In brief, cells were activated at 1×10^7 cells/ml. After 4 h of stimulation, cells were washed with ice-cold buffer (PBS with 0.5% BSA and 2 mM EDTA) and labeled with IL-10- or IFN-γ-specific or premixed (1:1) IFN-γ- and IL-10-specific capture matrix in cold medium for 5 min on ice. Subsequently, the labeled cells were diluted in prewarmed medium ($\leq 10^5$ cells/ml) and subjected to a 45-min cytokine secretion period at 37°C under slow continuous rotation. The cytokine secretion was stopped by filling up the tube with cold buffer and subsequent incubation on ice for 15 min. Cells were harvested and surface stained with specific detection Abs for IL-10 (allophycocyanin or PE) or IFN-γ (PE) or equal amounts of specific detection Abs for IL-10 (allophycocyanin or PE) and IFN-γ (PE or FITC). IL-10$^+$ and IL-10$^-$, or IFN-γ$^+$ and IFN-γ$^-$, or IFN-γ$^+$IL-10$^-$, IFN-γ$^+$IL-10$^+$, IFN-γ$^-$IL-10$^+$, and IFN-γ$^-$IL-10$^-$ Th cell subsets were purified (>95%) by cell sorting.

Cytometric bead array (CBA) assay

Supernatants from 48- or 72-h cultures of P/I- stimulated Th cell subpopulations were analyzed using the human Th1/Th2 cytokine CBA kit (BD Biosciences), which allows the simultaneous detection and quantification of soluble IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ in a single sample. In brief, a mixture of 10 μl of each of the six different bead suspensions specific for each cytokine (resolved in FL3 channel) was incubated with 50 μl of sample and 50 μl of PE-conjugated detection Ab (resolved in FL2 channel) for 3 h. Following acquisition of sample data by a FACSCalibur, the results were analyzed using the BD Biosciences CBA analysis software.

In vitro expansion and cytokine profiling of Th cell subsets

Sorted Th cell subsets were seeded in a 96-well plate (2×10^4 cells/well). Cytokines were added twice during the expansion. The cytokine profile was assessed at day 7, after 6 h of P/I stimulation (the last 4 h with brefeldin A) and by intracellular cytokine staining for IFN-γ and/or IL-10 and FACS analysis.

Real-time quantitative PCR analysis

Total RNA from the different Th cell subsets was extracted using the Absolutely RNA Microprep Kit (Stratagene) and reverse transcribed with TaqMan reverse transcription reagents (Roche Applied Biosystems) according to the manufacturer's recommendations. cDNA was analyzed for the expression of *IL10*, *IFNG*, *GATA3*, *Tbet*, *Sp1*, and ubiquitin (*UBCH5B*) by real-time PCR (primer sequences available upon request) using a LightCycler FastStart DNA Master SYBR GreenI Kit and a LightCycler (Roche Applied Science). Quantification of target gene expression was calibrated according to the values relative to the expression of ubiquitin gene.

Bisulphite treatment, PCR amplification, and semiquantitative DNA sequencing

Different Th cells were isolated from buffy coats of 30 healthy donors as described above. High molecular weight genomic DNA from each purified cell subset was isolated using a QIAamp DNA minikit (Qiagen) as recommended by the manufacturer. Three pools for each subset were analyzed. Each pool consisted of equal amounts of DNA from 10 age- and sex-matched (5 male and 5 female) donors. The average age of the donors are 39.1, 44.7, and 40.0 years for pools 1, 2, and 3, respectively. The DNA methylation status of pooled DNA was analyzed by direct bisulfite-modified DNA sequencing. The bisulfite treatment was performed as described earlier (40). Briefly, heat-denatured genomic DNA was embedded in 2% low-melting agarose (SeaPlaque; Biozym) and incubated for 4 h at 50°C in sodium disulfite solution (Merck) and then washed with Tris-EDTA buffer. Subsequently, PCR fragments were generated using the bisulfite-treated DNA as template. Primers were designed corresponding to the bisulfite-modified DNA sequence (primer sequences available upon request). PCR products were sequenced from both orientations with the dye terminator chemistry (Applied Biosystems V3.1.) using the PCR primers. The sequencing reaction was performed with an annealing temperature at 55°C and extension at 60°C for 24 cycles. Methylation levels were calculated using the Applied Biosystems trace files and previously described software (41, 42).

Chromatin immunoprecipitation assay (ChIP)

Sorted IL-10$^+$ and IL-10$^-$ Th cells were fixed with 1% formaldehyde for 10 min at room temperature. The fixation was stopped with 0.125 M glycine. The chromatin was sheared to 200–1000 bp in length by sonication with five pulses of 10 s at 30% power (Bandelin). The chromatin was incubated with Ab directed against hyperacetylated histone H3 or lysine 4 trimethylated histone H3 (Upstate Biotechnology) overnight, followed by incubation with protein A-MicroBeads (Miltenyi Biotec) for 2 h. Washing steps were performed on μ columns (Miltenyi Biotec) with high salt, low salt, LiCl, and Tris-EDTA buffer sequentially. Chromatin precipitate was eluted with 1% SDS and 0.1 M $NaHCO_3$. Cross-links were reversed by incubation at 65°C for 4 h in the presence of 0.2 M NaCl, and the DNA was purified with NucleoSpin Extract II (Macherey-Nagel). The amount of immunoprecipitated DNA was determined by real-time PCR with LightCycler (Roche Applied Science) using FASTStart SYBR Green Master (Roche Applied Science).The relative amount of DNA was calculated with $2^{(\text{crossing point input} - \text{crossing point input})}$.

Statistics

The two-tailed Welch *t* test was performed using GraphPad Prism 4.00. Values of $p \leq 0.05$ were regarded as significant. Data are presented as mean of replicates of relative mRNA expression from the same donor.

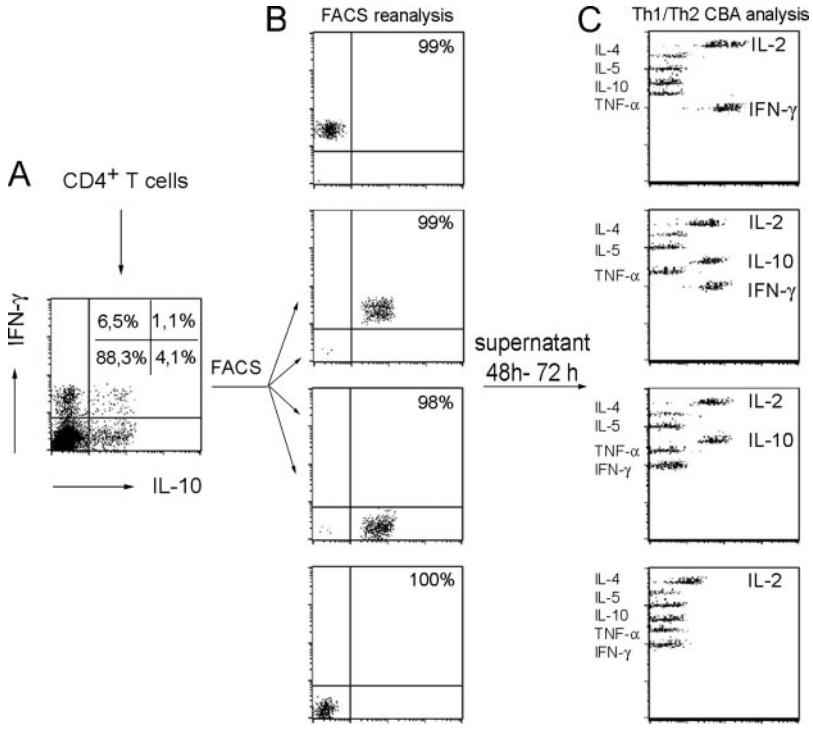


FIGURE 1. Isolation and characterization of human IL-10$^+$IFN-γ^-, IL-10$^+$IFN-γ^+, and IL-10$^-$IFN-γ^+ and IL-10$^-$IFN-γ^- Th cell subsets. *A*, Induction and analysis of CD4$^+$ Th cell subsets. CD4$^+$ T cells were stimulated with P/I followed by IL-10/IFN-γ secretion assay. The following Th cell subsets were identified: IL-10$^+$IFN-γ^-, IL-10$^+$IFN-γ^+, and IL-10$^-$IFN-γ^+, and IL-10$^-$IFN-γ^-. *B*, Purity analysis of CD4$^+$ Th cell subsets. The induced Th cell subsets were sorted by a FACSDiva. A small fraction of each sorted subset was reanalyzed for purity on a FACSCalibur. Similar detection and purification results were obtained in 46 independent experiments. *C*, Distinct cytokine profiles of CD4$^+$ Th cell subsets. Equal amounts of cells from each subset were cultured for 48 or 72 h. Supernatants were assessed for IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ (from *top* to *bottom*) by CBA assay and results were analyzed on a FACSCalibur. Data shown are representative of five separate experiments.

Results

Distinct cytokine profiles of IL-10$^+$IFN-γ^-, IL-10$^+$IFN-γ^+, and IL-10$^-$IFN-γ^+ and IL-10$^-$IFN-γ^- Th cell subsets

In our initial experiments, we observed that after both polyclonal stimulation with P/I or staphylococcal enterotoxin B and Ag-specific stimulation with CMV-derived Ags, human IL-10$^+$ Th cells can be delineated into a subset of only IL-10-secreting cells and a subset of Tr1-like IL-10- and IFN-γ-secreting cells (data not shown). To isolate ex vivo different human Th cell subsets secreting IL-10 or IFN-γ or both, we used a new technology, a double cytokine secretion assay for IFN-γ and IL-10. After stimulation of CD4$^+$ T cells with P/I, IL-10$^+$IFN-γ^- (1–5%), IL-10$^+$IFN-γ^+ (0.6–3%), IL-10$^-$IFN-γ^+ (5–15%), and IL-10$^-$IFN-γ^- (80–90%), Th cell subsets were induced (Fig. 1*A*) and sorted to a purity exceeding 95% in all experiments (Fig. 1*B*). To confirm the supposed cytokine secretion pattern of the isolated Th cell subsets, purified cells were cultured for 48 or 72 h and supernatants were analyzed for various cytokines. IL-10$^+$IFN-γ^- Th cells produced only IL-10 but no IFN-γ, while IL-10$^-$IFN-γ^+ Th cells produced only IFN-γ, but no IL-10. As expected, Tr1-like IL-10$^+$IFN-γ^+ Th cells secreted both cytokines, whereas IL-10$^-$IFN-γ^- Th cells secreted neither (Fig. 1*C*).

To further phenotypically analyze ex vivo IL-10-secreting Th cells, an IL-10 secretion assay was performed with CD45RO or CD45RA and CCR7-labeled CD4$^+$ cells. As characterized by expression of the CD45A and CD45RO isoforms and homing receptor CCR7, IL-10-secreting Th cells resembled Ag-experienced CD45RA$^-$CCR7$^+$ central memory Th cells (43) (Fig. 2).

mRNA expression in human IL-10-secreting Th cell subsets

We next performed quantitative real-time PCR analysis to assess mRNA expression of *IL10* and *IFNG* and to evaluate expression levels of transcription factors that have been associated with the regulation of *IL10* and *IFNG* such as *Sp1* (32), *FoxP3* (44) and *GATA3* (45–47), and *Tbet* (29). Abundant amounts of *IL10* mRNA were obtained only in IL-10-secreting subsets, IL-10$^+$IFN-γ^-, IL-10$^+$IFN-γ^+ Th cells. Notably, *GATA3* mRNA expression was significantly higher in IL-10$^+$IFN-γ^- Th cells ($p < 0.05$) in comparison to IL-10$^+$IFN-γ^+ Th cells. *Sp1* and *FoxP3* were not differentially expressed (data not shown). As expected, *IFNG* and higher *Tbet* mRNA expression levels were restricted to IFN-γ-secreting Th cells, except for one sample with lower expression in the IL-10$^+$IFN-γ^+ Th cells in comparison to the other four donors (Fig. 3). Together, these data demonstrate that *IL10* gene expression in human Th cells is regulated at the level of transcription.

Epigenetic status of the IL10 *gene*

We next assessed the level of DNA methylation of the entire *IL10* gene by DNA methylation in CD4$^+$ Th cell subsets purified according to IL-10 and/or IFN-γ secretion. Fig. 4*A* illustrates a schematic map of the 18.1-kb *IL10* gene locus spanning 9.1 kb upstream and 9 kb downstream of the transcriptional start site. Thirteen regions of interest (ROI) were analyzed, encompassing 88 CpGs. ROI were selected preferentially in conserved sequences (man and mouse), with priority given to known regulatory regions (promoter, HSS) and CpG density. There are no CpG islands

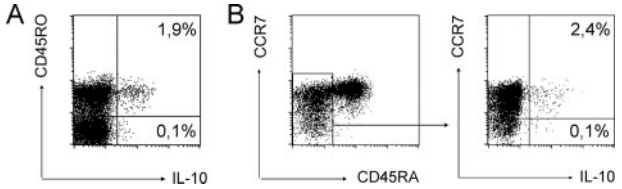


FIGURE 2. Phenotypic analysis of primary IL-10-secreting Th cells. *A*, IL-10-secreting Th cells are enriched in CD45RO$^+$ memory compartment. IL-10 secretion assay was performed with CD45RO-labeled CD4$^+$ Th cells before P/I stimulation. *B*, Ex vivo IL-10-secreting Th cells resemble Ag-experienced CD45RA$^-$CCR7$^+$ central memory cells. IL-10 secretion assay was performed with CD45RA- and CCR7-labeled CD4$^+$ Th cells before P/I stimulation. The data shown are representative of three independent experiments.

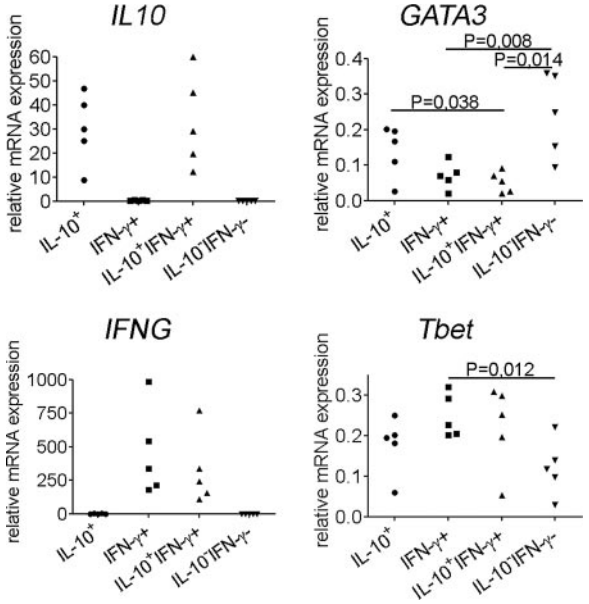


FIGURE 3. mRNA expression by IL-10$^+$IFN-γ^-, IL-10$^+$IFN-γ^+, IL-10$^-$IFN-γ^+, and IL-10$^-$IFN-γ^- Th cell subsets. *IL10*, *IFNG*, and transcription factors *GATA3* and *Tbet* mRNA levels in ex vivo-differentiated CD4$^+$ Th cell subsets from a same donor (as described in Fig. 1, *A* and *B*) were quantified by real-time PCR and normalized to human E2 ubiquitin-conjugating enzyme (*UBCH5B*) mRNA levels. The data shown are derived from five independent experiments.

within this locus as predicted by a cpgplot program (http://www.ebi.ac.uk/emboss/cpgplot/). Due to the low frequency of IL-10$^+$ Th cells and to increase uniformity of all measurements, the same amounts of DNA of each Th cell subset (Fig. 1, *A* and *B*) from peripheral blood samples of 30 healthy donors were pooled and subjected to bisulfite conversion, PCR amplification, and DNA sequencing.

Strikingly, no methylation pattern specific for IL-10-secreting Th cells could be demonstrated as compared with IL-10-nonsecreting Th cells. Two CpGs of ROI 1, located 9.1 kb upstream of the transcriptional start site, showed low levels of methylation in both IL-10$^+$ and IL-10$^-$ Th cells. CpGs of ROI 2 and 3 (5.5 and 5 kb upstream) and ROI 12 (3′ end) were almost fully methylated in all cell subsets. CpGs of ROI 4 (2.5 kb upstream) displayed alternating patterns of methylation, without an explicit preference assignable to a certain Th cell subset. Two CpGs in ROI 5 (proximal promoter) were slightly demethylated in IL-10$^+$ vs IL-10$^-$ Th cells. There was no similar methylation pattern observed for adjacent CpGs. In contrast to the *IL10* loci conservation, the two CpGs in ROI 5 were not evolutionary conserved between man and mouse. Some CpG sites of ROI 6 (intron I), 7 (intron I and exon II), 8 (exon III), 9 (intron IV), 10 (intron IV and exon V), 11 (3′ untranslated region), and 13 (3′ end) showed low levels of methylation throughout all Th cell subsets analyzed (Fig. 4*B*). Similar results were obtained for IL-10-secreting Th cells isolated from in vitro-established human Th1 and Th2 clones (Fig. 4*C*). Further analysis on histone modifications by histone 3 acetylation (H3Ac; Fig. 4*D*) and histone 3 lysine 4 trimethylation (H3K4me3; Fig. 4*E*), which closely correlate with transcriptional activity (48), showed that H3Ac and H3K4me3 associated with activation of *IL10* not only in ROI 5, but also in ROI 1, 2, 4, and 13 to a lesser extent. Taken together, these data indicate that limited differential demethylation of only two CpGs at the proximal promoter correlated with *IL10* gene expression. However, there is no methylation pattern correlating with *IL10* gene expression in any other selected

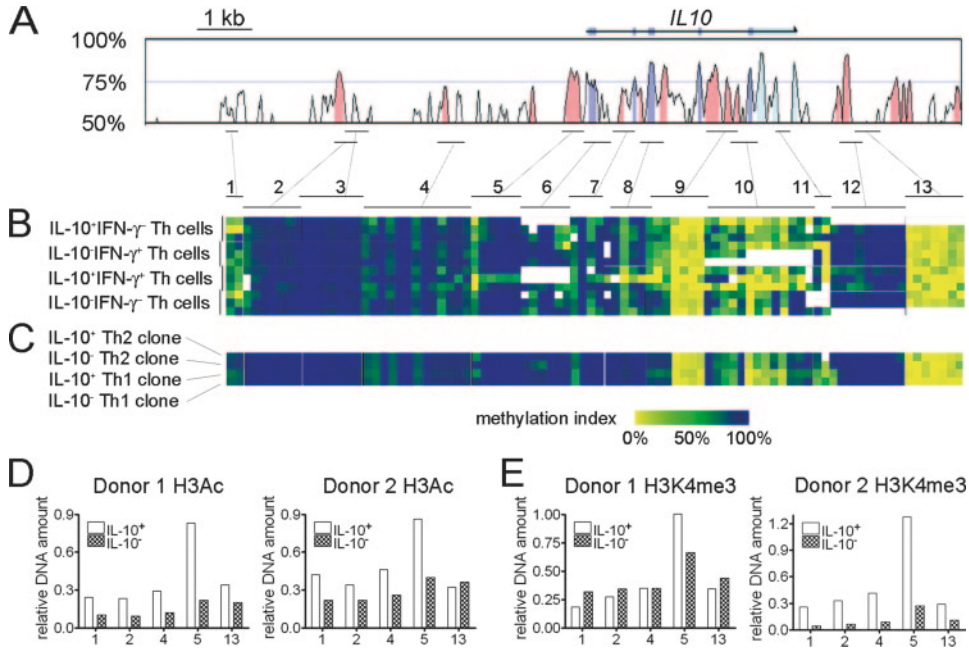


FIGURE 4. Genomic organization and quantitative DNA methylation and histone modification analyses of the human *IL10* gene. *A*, Genomic organization of the human *IL10* gene and alignment of human and mouse *IL10* gene loci with DNA sequence identity >50% over at least 100 bp are shown in the histogram plot (59). The selected ROI are labeled below the gene locus. *B*, The DNA methylation status of three pooled DNA populations of each IL-10$^+$IFN-γ^-, IL-10$^+$IFN-γ^+, IL-10$^-$IFN-γ^+ and IL-10$^-$IFN-γ^- Th cell subsets and IL-10$^+$ and IL-10$^-$ cells from Th1 and Th2 clones (*C*) are shown in rows. DNA methylation levels were determined by signal proportions between C and T peaks in colors shown in columns. Both peaks represent the average signal of all DNA copies generated during the PCR. Each rectangle in the grid represents a distinct CpG site on the designated ROI. ChIP assay assessing H3Ac (*D*) and H3K4me3 (*E*) at indicated ROI in ex vivo-purified IL-10$^+$ and IL-10$^-$ Th cells. Immunoprecipitated DNA was quantified by real-time PCR and normalized to input. The data shown are from two of three independent experiments.

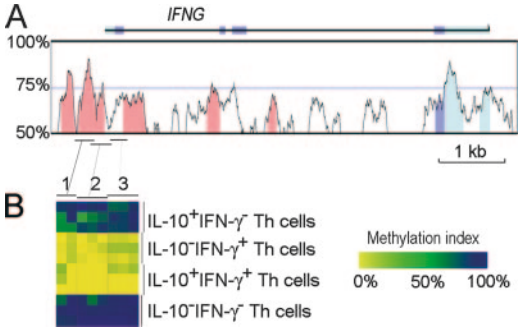


FIGURE 5. Correlation between hypomethylation and IFN-γ expression in the *IFNG* gene promoter, exon I, and intron I in human IFN-γ-secreting vs nonsecreting Th cells. *A*, Genomic organization of the human *IFNG* gene and alignment of human and mouse *IFNG* gene loci with DNA sequence identity >50% over at least 100 bp are shown in the histogram plot. The selected ROI are labeled below the gene locus. Overlapping ROI 1, 2, and 3 in the proximal promoter, exon 1, and intron 1 are depicted below the gene locus. ROI 1 consists of CpG sites of −295 and −186; ROI 2, −186, −54 and +122; and ROI 3, −54, +122 and +128 relative to the transcriptional start site. *B*, Cell types tested and method of CpG methylation quantification are the same as in Fig. 4*B*. These hypomethylation and hypermethylation patterns were reproducible in IFN-γ^+ and IFN-γ^- Th cells isolated from at least four individual donors, respectively.

ROI of the *IL10* locus, regardless of whether evolutionary conserved coding or noncoding regions were analyzed.

Hypomethylation pattern of the IFNG *gene promoter in IL-10$^+$IFN-γ$^+$ and IL-10$^-$IFN-γ$^+$ Th cell subsets*

For comparison, we investigated the methylation status of the *IFNG* gene focusing on the promoter region in these Th cell populations. IFN-γ^+ Th cell subsets (IL-10$^+$IFN-γ^+ and IL-10$^-$IFN-γ^+ cells) were hypomethylated at the *IFNG* locus compared with IFN-γ-nonsecreting Th cell subsets (IL-10$^+$IFN-γ^- and IL-10$^-$IFN-γ^- cells; Fig. 5). Particularly, CpG sites at positions −295, −186, −54, +122, and +128 relative to the start of transcription were hypomethylated specifically in IFN-γ^+ Th cells. Our data emphasize the strong correlation between expression of the *IFNG* gene and hypomethylation of its promoter.

Limited IL-10 re-expression in ex vivo-expanded IL-10$^+$ Th cell subsets

To determine a possible functional relevance of the lack of epigenetic memory of *IL10*, we analyzed the stability of IL-10 re-expression in comparison to IFN-γ re-expression in ex vivo-isolated IL-10$^+$ and IFN-γ^+ Th cell subsets following short-term in vitro cultures. One week after isolation and expansion under neutral conditions with rIL-7 plus rIL-15 (rIL-7/15), re-expression of IL-10 and IFN-γ was assessed after restimulation with P/I. Little induction of IFN-γ was observed in cultured IL-10$^+$IFN-γ^- and IL-10$^+$ Th cell subsets, most likely due to a small fraction of pre-Th1 cells (49) in response to homeostatic cytokines rIL-7/15 (Fig. 6*A*). Strikingly, from both sorted IL-10$^+$IFN-γ^- (Fig. 6*A*) and IL-10$^+$IFN-γ^+ (Fig. 6*B*) Th cells, only ~10% re-expressed IL-10. In contrast, in both IFN-γ-secreting subsets, IL-10$^+$IFN-γ^+ (Fig. 6*B*) and IL-10$^-$IFN-γ^+ (Fig. 6*C*) Th cells, >90% of the cells maintained IFN-γ expression. To address whether stable IL-10 re-expression is restricted to a special subset of Th cells, IL-10$^+$IFN-γ^- Th cells were reisolated following 1 wk of culture of IL-10$^+$IFN-γ^- Th cells. After 1 wk of further expansion and stimulation of these reisolated IL-10$^+$IFN-γ^- cells, again only ~10% of cells re-expressed IL-10 (Fig. 6*A*). Thus, 99% of ex vivo-isolated human IL-10$^+$ Th cells were unable to maintain IL-10 expression upon secondary restimulation, implicating that the immunoregulatory cytokine IL-10 is excluded from the functional cytokine memory of human memory Th cells compared with the effector cytokine IFN-γ.

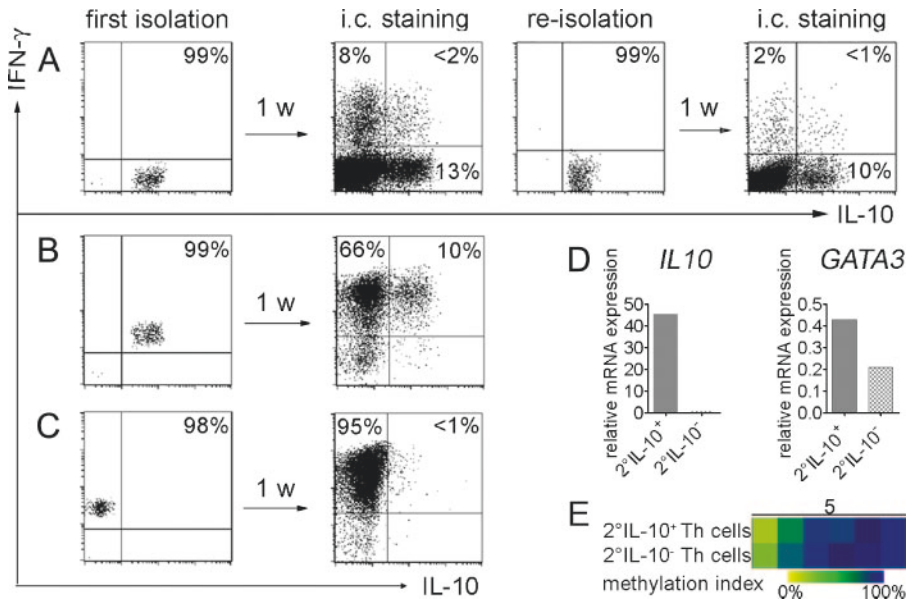


FIGURE 6. Attenuation of IL-10 re-expression. Ex vivo IL-10$^+$IFN-γ^- (*A*), IL-10$^+$IFN-γ^+ (*B*), and IL-10$^-$IFN-γ^+ (*C*) Th cell subsets were sorted as described in Fig. 1. Purified Th cell subsets were expanded under neutral (rIL-7 plus rIL-15) conditions. After 1 wk, a second-round cytokine secretion assay for IL-10 and IFN-γ was performed to reisolate IL-10$^+$IFN-γ^- cells of first-round sorted IL-10$^+$IFN-γ^- cells. In parallel, re-expression of IL-10 and IFN-γ was analyzed by intracellular staining (i.c.) following 6 h of P/I restimulation for all of the subsets. Similar results were obtained in three independent experiments. *D*, Kinetics of *IL10* and *GATA3* expression. IL-10$^+$ and IL-10$^-$ Th cell subsets were reisolated after 1 wk of culture of IL-10$^+$ cells and subjected to real-time PCR analysis. Normalization of *IL10* and *GATA3* expression was the same as in Fig. 3. One of two independent experiments is shown. *E*, DNA methylation status of the promoter region of the *IL10* gene (ROI 5) in secondary (2°) IL-10$^+$ and IL-10$^-$ cell subsets. Method of CpG methylation quantification is the same as described in Fig. 4*B*. The data shown are representative of four independent experiments.

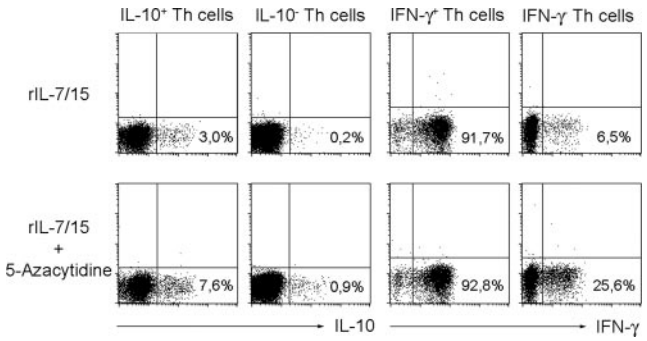


FIGURE 7. Dnmts inhibitor 5-azacytidine treatment of primary IL-10-secreting and nonsecreting Th cells vs IFN-γ-secreting and nonsecreting Th cells. IL-10$^+$ and IL-10$^-$, IFN-γ$^+$ and IFN-γ$^-$ Th cell subsets were highly purified following IL-10 and IFN-γ secretion, respectively. rIL-7/15 or rIL-7/15 plus 5-azacytidine were applied to cultures at days 0 and 2. At day 7, re-expression of cytokine was assessed by P/I stimulation for 6 h in the presence of brefeldin A for the last 4 h. The data shown are representative of three independent experiments.

Transcriptional regulation of secondary IL-10-producing Th cells

To understand the molecular mechanisms underlying the inefficiency of functional IL-10 memory, we next performed quantitative real-time PCR analysis to examine mRNA expression of *IL10* and *GATA3* by short-term cultured cells. IL-10$^+$ and IL-10$^-$ cells were reisolated after 1 wk of culture of IL-10$^+$ Th cells. As expected and similar to the results observed in primary Th cell subsets (Fig. 3), *IL10* mRNA expression was restricted to IL-10-secreting Th cells (Fig. 6*D*). Also, a higher level of *GATA3* was detected only in IL-10-secreting Th cells (Fig. 6*D*). The kinetics of *IL10* and *GATA3* expression suggest that *IL10* gene expression is regulated at the level of transcription and GATA-3 is important with regard to the sustained *IL10* gene expression. To exclude the possibility that the lack of IL-10 re-expression could be due to reversible alterations in DNA methylation status, we compared the level of DNA methylation of ROI 3 (upstream), 5 (promoter), and 9 (intron IV) in reisolated IL-10$^+$ vs IL-10$^-$ Th cell subsets. The detected methylation patterns of secondary IL-10$^+$ and IL-10$^-$ cells resembled those of primary Th cell subsets (Figs. 4*B* and 6*E*, and data not shown).

5-Azacytidine not only induces IL-10 expression in IL-10$^-$ cells but also increases IL-10 re-expression in cultured IL-10$^+$ cells

We next investigated whether *IL10* gene expression can be in principle regulated by DNA methylation. To this end, 5-azacytidine was applied to IL-10$^+$ and IL-10$^-$ and IFN-γ$^+$ and IFN-γ$^-$ Th cell cultures. 5-Azacytidine is an analog of cytidine that inhibits DNA methyltransferases (Dnmts) during replication, thereby changing DNA methylation status and the expression of genes silenced by methylation (50). 5-Azacytidine treatment led to not only a 4.5-fold induction of IL-10 production by primary IL-10$^-$ cells, but also a 2.5-fold increase of IL-10 re-expression in primary IL-10$^+$ cells, compared with cells cultured in the absence of 5-azacytidine, respectively (Fig. 7). In agreement with previous finding (51), 5-azacytidine treatment resulted in a 4-fold induction of IFN-γ production in primary IFN-γ$^-$ cells. As expected, this treatment did not increase the IFN-γ production in primary IFN-γ$^+$ cells (Fig. 7). These data suggest that like *IFNG* gene expression can be regulated by DNA methylation, *IL10* gene expression can be in principle regulated by DNA methylation as well.

Discussion

Epigenetic modification of effector cytokine genes such as *Ifng*/*IFNG* and *Il4* have been demonstrated in Th cells by changes in DNA methylation, histone de/acetylation, and rearrangement of the chromatin within the nucleus. The "poised" state apparent in effector/memory Th cells is thought to allow for rapid secretion of effector cytokines when rechallenged by invading pathogens (52–54). In this study, we show that things are different for the immunoregulatory cytokine IL-10. IL-10-secreting memory Th cells isolated ex vivo did not display a specific DNA methylation pattern as compared with Th cells not secreting IL-10. In contrast, hypomethylation of the *IFNG* gene promoter strongly correlated with IFN-γ expression in memory Th cells. In accordance with the lack of methylation memory, the majority of ex vivo-isolated IL-10-secreting Th cells lack a functional memory for IL-10 re-expression after restimulation. The unique role of IL-10 with its broad immunoregulatory functions might be a major reason for these differences in expression and regulation. So far, the regulation of *IL10* gene expression, particularly in human Th cells representing a major source of IL-10 in the course of adaptive immune responses had remained poorly understood.

Unlike memory Th cell subsets producing effecter cytokines such as IFN-γ and IL-4, efficient protocols for the generation of IL-10-secreting cells from naive Th cells have not been established yet or results remain controversial. Moreover, in vivo-generated human IL-10-secreting Th cells are usually rare and cannot be assessed according to characteristic surface markers. Using a new technology, we isolated human Th cell subsets secreting IL-10 or IFN-γ, or both, directly ex vivo from peripheral blood of healthy donors after short-term polyclonal stimulation. To evaluate epigenetic mechanisms that underlie *IL10* gene expression, we provide here the first assessments of DNA methylation status of the *IL10* gene, encompassing 88 selected CpGs, and the *IFNG* gene promoter, encompassing 5 CpGs. Our results offer initial evidence that in contrast to the expression of the effector cytokine *IFNG*, the expression of immunoregulatory cytokine *IL10* is not mainly regulated by DNA methylation. Concomitantly, ex vivo IL-10-secreting Th cells lack a functional cytokine memory for IL-10 re-expression after short-term in vitro expansion, as compared with the epigenetically marked *IFNG* in IFN-γ-secreting Th cells.

Demethylation of cytosine in CpG dinucleotides in regulatory regions often correlates with other epigenetic modifications such as increased nuclease sensitivity. DNase I HSS, are believed to reflect the "open" chromatin configuration (55). With respect to the regulation of *Il10* gene expression, such DNase I HSS have been described in macrophages, dendritic cells, and Th1 and Th2 clones (34, 38, 56). One HSS described as Th2 specific and constitutive coexists with DNA methylation in the promoter region (38) that is similar to our ROI 5. At the level of histone modifications, the relative hyper-H3Ac and H3K4me3 do not completely match the DNA methylation status. It seems that a slight coincidence between H3Ac and H3K4me3 and DNA methylation only exists in the ROI 5 (Fig. 4, *D* and *E*), suggesting an intricate interplay between different layers of chromatin modifications given by histone acetylation and methylation and DNA methylation that contribute to the regulation of *IL10* gene expression.

Of note, GATA-3 (47) that has been implicated in the regulation of *Il10* gene expression (45, 46, 57) was significantly up-regulated in IL-10$^+$IFN-γ$^-$ vs IL-10$^+$IFN-γ$^+$ Th cells. Furthermore, *GATA3* remained up-regulated in reisolated IL-10$^+$ Th cells in comparison to IL-10$^-$ Th cells following 1-wk culture of primary IL-10$^+$ Th cells (Fig. 6*D*), confirming the importance of GATA-3

with regard to *IL10* gene expression. Conversely, Sp1 is ubiquitously expressed (58) and we did not find a correlation between *Sp1* and *IL10* gene expression (data not shown). However, we currently cannot exclude the possibility that the limited difference in DNA methylation is conferring transcriptional specificity for undefined transcription factors in human Th cells.

In accordance with the lack of epigenetic memory of *IL10* by DNA methylation, human IL-10-secreting Th cells displayed a limited memory for IL-10 re-expression after short-term in vitro culture. In contrast, IFN-γ-secreting Th cell subsets were characterized by a specific epigenetic memory for *IFNG* gene expression. However, when treated with DNA Dnmts inhibitor 5-azacytidine, IL-10 expression was significantly augmented in both ex vivo IL-10^+ and IL-10^- Th cells after 1 wk of culture to a similar extent as augmented IFN-γ production in treated ex vivo IFN-γ-nonproducers (Fig. 7). Although this shows that in principle expression of the *IL10* gene can be subject to regulation by demethylation, the *IL10* gene in IL-10^+ Th cells directly isolated ex vivo (Fig. 4*B*) in in vitro-established IL-10^+ Th cell clones (Fig. 4*C*) and in IL-10^+ Th cells reisolated from in vitro cultures initiated with IL-10^+ Th cells (Fig. 6*E*) is not imprinted by demethylation, in contrast to the *IFNG* gene (Fig. 5*B*). Apparently, the expression of the major immunoregulatory cytokine *IL10* in human memory Th cells is regulated in a completely different manner compared with an effector cytokine such as *IFNG*. *IL10* gene expression might be determined by a variable mixture of signals rather than by a fixed heritable program acquired during Th cell differentiation as seen for effector cytokines. An epigenetic memory for *IL10* gene expression in memory Th cells generated in immune responses specific for exogenous Ags could be dangerous, because it could allow for an immediate and undesired suppression of an immune response.

In summary, in contrast to effector cytokines such as IFN-γ, IL-10 is excluded from the functional cytokine memory of human memory Th cells. Our results indicate that cytokine expression in memory Th cells is differentially regulated for the major immunoregulatory cytokine IL-10 and the effector cytokine IFN-γ, most likely to ensure efficient pathogen-specific recall immune responses.

Acknowledgments

We thank Drs. J. L. Grogan, M. Bros, and J. Li for valuable comments and S. Cottrell and F. Hatam for critical reading of this manuscript. We are grateful to K. Raba, T. Kaiser, L. Reiners-Schramm, R. Cortese, and J. Dietrich for their expert technical assistance.

Disclosures

The authors have no financial conflict of interest.

References

1. Bluestone, J. A., and A. K. Abbas. 2003. Natural versus adaptive regulatory T cells. *Nat. Rev. Immunol.* 3: 253–257.
2. Sakaguchi, S., N. Sakaguchi, M. Asano, M. Itoh, and M. Toda. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α-chains (CD25): breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* 155: 1151–1164.
3. Groux, H., A. O'Garra, M. Bigler, M. Rouleau, S. Antonenko, J. E. de Vries, and M. G. Roncarolo. 1997. A $CD4^+$ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389: 737–742.
4. Chen, Y., V. K. Kuchroo, J. Inobe, D. A. Hafler, and H. L. Weiner. 1994. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science* 265: 1237–1240.
5. Moore, K. W., R. de Waal Malefyt, R. L. Coffman, and A. O'Garra. 2001. Interleukin-10 and the interleukin-10 receptor. *Annu. Rev. Immunol.* 19: 683–765.
6. Kuhn, R., J. Lohler, D. Rennick, K. Rajewsky, and W. Muller. 1993. *Interleukin-10*-deficient mice develop chronic enterocolitis. *Cell* 75: 263–274.
7. Ding, W., S. Beissert, L. Deng, E. Miranda, C. Cassetty, K. Seiffert, K. L. Campton, Z. Yan, G. F. Murphy, J. A. Bluestone, and R. D. Granstein. 2003. Altered cutaneous immune parameters in transgenic mice overexpressing viral *IL-10* in the epidermis. *J. Clin. Invest.* 111: 1923–1931.
8. Lang, R., R. L. Rutschman, D. R. Greaves, and P. J. Murray. 2002. Autocrine deactivation of macrophages in transgenic mice constitutively overexpressing *IL-10* under control of the human CD68 promoter. *J. Immunol.* 168: 3402–3411.
9. Murray, P. J., L. Wang, C. Onufryk, R. I. Tepper, and R. A. Young. 1997. T cell-derived IL-10 antagonizes macrophage function in mycobacterial infection. *J. Immunol.* 158: 315–321.
10. Pinderski, L. J., M. P. Fischbein, G. Subbanagounder, M. C. Fishbein, N. Kubo, H. Cheroutre, L. K. Curtiss, J. A. Berliner, and W. A. Boisvert. 2002. Overexpression of *interleukin-10* by activated T lymphocytes inhibits atherosclerosis in LDL receptor-deficient mice by altering lymphocyte and macrophage phenotypes. *Circ. Res.* 90: 1064–1071.
11. Asseman, C., S. Mauze, M. W. Leach, R. L. Coffman, and F. Powrie. 1999. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J. Exp. Med.* 190: 995–1004.
12. Barrat, F. J., D. J. Cua, A. Boonstra, D. F. Richards, C. Crain, H. F. Savelkoul, R. de Waal-Malefyt, R. L. Coffman, C. M. Hawrylowicz, and A. O'Garra. 2002. In vitro generation of interleukin 10-producing regulatory $CD4^+$ T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. *J. Exp. Med.* 195: 603–616.
13. Jonuleit, H., E. Schmitt, G. Schuler, J. Knop, and A. H. Enk. 2000. Induction of interleukin 10-producing, nonproliferating $CD4^+$ T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. *J. Exp. Med.* 192: 1213–1222.
14. Kemper, C., A. C. Chan, J. M. Green, K. A. Brett, K. M. Murphy, and J. P. Atkinson. 2003. Activation of human $CD4^+$ cells with CD3 and CD46 induces a T-regulatory cell 1 phenotype. *Nature* 421: 388–392.
15. Belkaid, Y., C. A. Piccirillo, S. Mendez, E. M. Shevach, and D. L. Sacks. 2002. $CD4^+CD25^+$ regulatory T cells control *Leishmania major* persistence and immunity. *Nature* 420: 502–507.
16. Hara, M., C. I. Kingsley, M. Niimi, S. Read, S. E. Turvey, A. R. Bushell, P. J. Morris, F. Powrie, and K. J. Wood. 2001. IL-10 is required for regulatory T cells to mediate tolerance to alloantigens in vivo. *J. Immunol.* 166: 3789–3796.
17. de la Rosa, M., S. Rutz, H. Dorninger, and A. Scheffold. 2004. Interleukin-2 is essential for $CD4^+CD25^+$ regulatory T cell function. *Eur. J. Immunol.* 34: 2480.
18. Lohning, M., A. Richter, and A. Radbruch. 2002. Cytokine memory of T helper lymphocytes. *Adv. Immunol.* 80: 115–181.
19. Grogan, J. L., M. Mohrs, B. Harmon, D. A. Lacy, J. W. Sedat, and R. M. Locksley. 2001. Early transcription and silencing of cytokine genes underlie polarization of T helper cell subsets. *Immunity* 14: 205–215.
20. Jaenisch, R., and A. Bird. 2003. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat. Genet.* 33(Suppl.): 245.
21. Reiner, S. L. 2005. Epigenetic control in the immune response. *Hum. Mol. Genet.* 14: R41–R46.
22. Wilson, C. B., K. W. Makar, M. Shnyreva, and D. R. Fitzpatrick. 2005. DNA methylation and the expanding epigenetics of T cell lineage commitment. *Semin. Immunol.* 17: 105–119.
23. Agarwal, S., and A. Rao. 1998. Modulation of chromatin structure regulates cytokine gene expression during T cell differentiation. *Immunity* 9: 765–775.
24. Avni, O., D. Lee, F. Macian, S. J. Szabo, L. H. Glimcher, and A. Rao. 2002. Th cell differentiation is accompanied by dynamic changes in histone acetylation of cytokine genes. *Nat. Immunol.* 3: 643–651.
25. Fields, P. E., G. R. Lee, S. T. Kim, V. V. Bartsevich, and R. A. Flavell. 2004. Th2-specific chromatin remodeling and enhancer activity in the Th2 cytokine locus control region. *Immunity* 21: 865–876.
26. Grogan, J. L., Z. E. Wang, S. Stanley, B. Harmon, G. G. Loots, E. M. Rubin, and R. M. Locksley. 2003. Basal chromatin modification at the *IL-4* gene in helper T cells. *J. Immunol.* 171: 6672–6679.
27. Lee, D. U., O. Avni, L. Chen, and A. Rao. 2004. A distal enhancer in the *interferon-γ* (*IFN-γ*) locus revealed by genome sequence comparison. *J. Biol. Chem.* 279: 4802–4810.
28. Martins, G. A., A. S. Hutchins, and S. L. Reiner. 2005. Transcriptional activators of helper T cell fate are required for establishment but not maintenance of signature cytokine expression. *J. Immunol.* 175: 5981–5985.
29. Szabo, S. J., S. T. Kim, G. L. Costa, X. Zhang, C. G. Fathman, and L. H. Glimcher. 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 100: 655–669.
30. Young, H. A., P. Ghosh, J. Ye, J. Lederer, A. Lichtman, J. R. Gerard, L. Penix, C. B. Wilson, A. J. Melvin, M. E. McGurn, et al. 1994. Differentiation of the T helper phenotypes by analysis of the methylation state of the *IFN-γ* gene. *J. Immunol.* 153: 3603–3610.
31. Ziegler-Heitbrock, L., M. Lotzerich, A. Schaefer, T. Werner, M. Frankenberger, and E. Benkhart. 2003. IFN-α induces the human *IL-10* gene by recruiting both IFN regulatory factor 1 and Stat3. *J. Immunol.* 171: 285–290.
32. Brightbill, H. D., S. E. Plevy, R. L. Modlin, and S. T. Smale. 2000. A prominent role for Sp1 during lipopolysaccharide-mediated induction of the *IL-10* promoter in macrophages. *J. Immunol.* 164: 1940–1951.
33. Tone, M., M. J. Powell, Y. Tone, S. A. Thompson, and H. Waldmann. 2000. *IL-10* gene expression is controlled by the transcription factors Sp1 and Sp3. *J. Immunol.* 165: 286–291.
34. Saraiva, M., J. R. Christensen, A. V. Tsytsykova, A. E. Goldfeld, S. C. Ley, D. Kioussis, and A. O'Garra. 2005. Identification of a macrophage-specific chromatin signature in the *IL-10* locus. *J. Immunol.* 175: 1041–1046.
35. Kitani, A., I. Fuss, K. Nakamura, F. Kumaki, T. Usui, and W. Strober. 2003. Transforming growth factor (TGF)-β1-producing regulatory T cells induce

Smad-mediated interleukin 10 secretion that facilitates coordinated immunoregulatory activity and amelioration of TGF-β1-mediated fibrosis. *J. Exp. Med.* 198: 1179–1188.
36. Cao, S., J. Liu, L. Song, and X. Ma. 2005. The protooncogene c-Maf is an essential transcription factor for *IL-10* gene expression in macrophages. *J. Immunol.* 174: 3484–3492.
37. Wang, Z. Y., H. Sato, S. Kusam, S. Sehra, L. M. Toney, and A. L. Dent. 2005. Regulation of *IL-10* gene expression in Th2 cells by Jun proteins. *J. Immunol.* 174: 2098–2105.
38. Im, S. H., A. Hueber, S. Monticelli, K. H. Kang, and A. Rao. 2004. Chromatin-level regulation of the *IL10* gene in T cells. *J. Biol. Chem.* 279: 46818–46825.
39. Jones, E. A., and R. A. Flavell. 2005. Distal enhancer elements transcribe intergenic RNA in the *IL-10* family gene cluster. *J. Immunol.* 175: 7437–7446.
40. Olek, A., J. Oswald, and J. Walter. 1996. A modified and improved method for bisulphite based cytosine methylation analysis. *Nucleic Acids Res.* 24: 5064–5066.
41. Lewin, J., A. O. Schmitt, P. Adorjan, T. Hildmann, and C. Piepenbrock. 2004. Quantitative DNA methylation analysis based on four-dye trace data from direct sequencing of PCR amplificates. *Bioinformatics* 20: 3005–3012.
42. Rakyan, V. K., T. Hildmann, K. L. Novik, J. Lewin, J. Tost, A. V. Cox, T. D. Andrews, K. L. Howe, T. Otto, A. Olek, et al. 2004. DNA methylation profiling of the human major histocompatibility complex: a pilot study for the human epigenome project. *PLoS Biol.* 2: E405.
43. Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401: 708–712.
44. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299: 1057–1061.
45. Pai, S. Y., M. L. Truitt, and I. C. Ho. 2004. *GATA-3* deficiency abrogates the development and maintenance of T helper type 2 cells. *Proc. Natl. Acad. Sci. USA* 101: 1993–1998.
46. Shoemaker, J., M. Saraiva, and A. O'Garra. 2006. GATA-3 directly remodels the *IL-10* locus independently of IL-4 in CD4$^+$ T cells. *J. Immunol.* 176: 3470–3479.
47. Zheng, W., and R. A. Flavell. 1997. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* 89: 587–596.
48. Shahbazian, M. D., and M. Grunstein. 2007. Functions of site-specific histone acetylation and deacetylation. *Annu. Rev. Biochem.* 76: 26.1–26.26.
49. Rivino, L., M. Messi, D. Jarrossay, A. Lanzavecchia, F. Sallusto, and J. Geginat. 2004. Chemokine receptor expression identifies pre-T helper (Th)1, pre-Th2, and nonpolarized cells among human CD4$^+$ central memory T cells. *J. Exp. Med.* 200: 725–735.
50. Christman, J. K. 2002. 5-Azacytidine and 5-aza-2′-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy. *Oncogene* 21: 5483–5495.
51. Fitzpatrick, D. R., K. M. Shirley, L. E. McDonald, H. Bielefeldt-Ohmann, G. F. Kay, and A. Kelso. 1998. Distinct methylation of the *interferon* γ (*IFN-*γ) and *interleukin 3* (*IL-3*) genes in newly activated primary CD8$^+$ T lymphocytes: regional *IFN-*γ promoter demethylation and mRNA expression are heritable in CD44^{high}CD8$^+$ T cells. *J. Exp. Med.* 188: 103–117.
52. Ansel, K. M., I. Djuretic, B. Tanasa, and A. Rao. 2006. Regulation of Th2 differentiation and *Il4* locus accessibility. *Annu. Rev. Immunol.* 24: 607–656.
53. Grogan, J. L., and R. M. Locksley. 2002. T helper cell differentiation: on again, off again. *Curr. Opin. Immunol.* 14: 366–372.
54. Lee, G. R., S. T. Kim, C. G. Spilianakis, P. E. Fields, and R. A. Flavell. 2006. T helper cell differentiation: regulation by *cis* elements and epigenetics. *Immunity* 24: 369–379.
55. Gross, D. S., and W. T. Garrard. 1988. Nuclease hypersensitive sites in chromatin. *Annu. Rev. Biochem.* 57: 159–197.
56. Kang, K. H., and S. H. Im. 2005. Differential regulation of the *IL-10* gene in Th1 and Th2 T cells. *Ann. NY Acad. Sci.* 1050: 97–107.
57. Chang, H. D., C. Helbig, L. Tykocinski, S. Kreher, J. Koeck, U. Niesner, and A. Radbruch. 2007. Expression of IL-10 in Th memory lymphocytes is conditional on IL-12 or IL-4, unless the *IL-10* gene is imprinted by GATA-3. *Eur. J. Immunol.* 37: 807–817.
58. Li, L., S. He, J. M. Sun, and J. R. Davie. 2004. Gene regulation by Sp1 and Sp3. *Biochem. Cell Biol.* 82: 460–471.
59. Bray, N., I. Dubchak, and L. Pachter. 2003. AVID: A global alignment program. *Genome Res.* 13: 97–102.

European Journal of Immunology

Loss of methylation at the *IFNG* promoter and CNS-1 is associated with the development of functional IFN-γ memory in human CD4$^+$ T lymphocytes

Jun Dong[1,2], Hyun-Dong Chang[2], Claudia Ivascu[3], Yu Qian[1], Soheila Rezai[4], Anna Okhrimenko[1,2], Lorenzo Cosmi[5], Laura Maggi[5], Florian Eckhardt[6], Peihua Wu[7], Joachim Sieper[7], Tobias Alexander[8], Francesco Annunziato[5], Manfred Gossen[9,10], Jun Li[11], Andreas Radbruch[2] and Andreas Thiel [1,2]

[1] Regenerative Immunology and Aging, Berlin-Brandenburg Center for Regenerative Therapies, Berlin, Germany
[2] Cell Biology Group, German Rheumatism Research Center Berlin, Berlin, Germany
[3] Epigenomics AG, Berlin, Germany
[4] Dipartimento di Clinica e Terapia Medica Applicata, Sezione di Reumatologia, Sapienza Università di Roma, Rome, Italy
[5] Center of Research, Transfer, High Education DENOTHE, University of Florence, Firenze, Italy
[6] B·R·A·H·M·S GmbH, Hennigsdorf, Germany
[7] Department of Gastroenterology, Infectiology and Rheumatology, Charité Universitätsmedizin Berlin, Berlin, Germany
[8] Department of Rheumatology and Clinical Immunology, Charité Universitätsmedizin Berlin, Berlin, Germany
[9] Max Delbrück Center for Molecular Medicine (MDC), Berlin, Germany
[10] Genetic Engineering, Berlin-Brandenburg Center for Regenerative Therapies, Berlin, Germany
[11] Center for Cardiovascular Research and Institute of Pharmacology, Charité-Universitätsmedizin Berlin, Berlin, Germany

Cytokine memory for IFN-γ production by effector/memory Th1 cells plays a key role in both protective and pathological immune responses. To understand the epigenetic mechanism determining the ontogeny of effector/memory Th1 cells characterized by stable effector functions, we identified a T-cell-specific methylation pattern at the *IFNG* promoter and CNS-1 in ex vivo effector/memory Th1 cells, and investigated methylation dynamics of these regions during the development of effector/memory Th1 cells. During Th1 differentiation, demethylation occurred at both the promoter and CNS-1 regions of *IFNG* as early as 16 h, and this process was independent of cell proliferation and DNA synthesis. Using an IFN-γ capture assay, we found early IFN-γ-producing cells from 2-day differentiating cultures acquired "permissive" levels of demethylation and developed into effector/memory Th1 cells undergoing progressive demethylation at the *IFNG* promoter and CNS-1 when induced by IL-12. Methylation levels of these regions in effector/memory Th1 cells of peripheral blood from rheumatoid arthritis patients correlated inversely with reduced frequencies of IFN-γ-producers, coincident with recruitment of effector/memory Th1 cells to the site of inflammation. Thus, after termination of TCR stimulation, IL-12 signaling potentiates the stable functional IFN-γ memory in effector/memory Th1 cells characterized by hypomethylation at the *IFNG* promoter and CNS-1.

Keywords: Demethylation · Human *IFNG* gene · IFN-γ cytokine memory · Promoter and CNS-1 · Th1 cell differentiation

Additional supporting information may be found in the online version of this article at the publisher's web-site

Correspondence: Dr. Jun Dong
e-mail: dong@drfz.de

Introduction

A unique feature of adaptive immunity is the generation of effector/memory T cells after primary activation that control pathogens and mediate effective protection during secondary challenges [1]. Depending on the stimuli and cytokine environment experienced during activation, naive T cells make decisions to proliferate and differentiate into Th1, Th2, or Th17 lineages [2]. When challenged by intracellular pathogens, naïve $CD4^+$ T cells can adopt a proinflammatory cell fate and differentiate into effector and memory Th1 cells, which are characterized by the production of the signature cytokine IFN-γ. Th1 cells play a key role in intracellular pathogen killing and exert proinflammatory effects in organ-specific autoimmune diseases [3]. During primary activation, the expression of the *Ifng/IFNG* gene in naïve $CD4^+$ T cells is initiated by TCR signaling, in conjunction with instructive IL-12 signaling through lineage-specific transcription factors (such as T-bet and the IL-12-responsive transcription factor STAT4, respectively) [4–6]. After termination of antigen stimulation, IL-12 also controls *Ifng* reactivation in effector/memory Th1 cells through STAT4 and T-bet [7]. During secondary activation, TCR signaling in the absence of the original instructive signal is sufficient to trigger rapid *Ifng/IFNG* reactivation in effector/memory Th1 cells. This functional cytokine memory for IFN-γ is achieved through a transcription factor network that engages the "poised" epigenetically imprinted regulatory regions in the *Ifng/IFNG* gene established during primary activation [8].

The accessibility of DNA binding sites to transcription factors is regulated by epigenetic modifications, including DNA methylation and covalent histone tail modifications [9]. Several studies have demonstrated an essential role of DNA methylation in determining the capacity of T cells to express *Ifng/IFNG*. For example, DNA methylation of a few specific CpGs in the *IFNG/Ifng* promoter is inversely correlated with *IFNG/Ifng* expression [10, 11]. We have also previously shown that treatment with DNA methylation inhibitors augments IFN-γ production, even in purified cells that do not originally produce IFN-γ [11]. In addition, de novo methylation of the *IFNG* promoter leads to reduced IFN-γ production in T cells infected with human immunodeficiency virus type I [12].

Analyses of regulatory elements in the *Ifng/IFNG* gene have provided considerable insight into the control of selective Th1 expression. For example, a conserved element termed CNS-1 (conserved noncoding sequence 1) located 5 kb upstream of the murine *Ifng* gene and 4.2 kb upstream of the human *IFNG* gene has been identified by sequence homology analysis between mice and humans. CNS-1 displays Th1-specific DNase I hypersensitivity and enhancer activity. The transcription factors NFAT and T-bet bind to this region in stimulated Th1-cell lines and augment its enhancer activity [13]. A minimal 500 bp stretch of the proximal *IFNG* promoter has been demonstrated to confer Th1/Tc1 selective expression [14, 15]. However, it remains to be elucidated how changes in the methylation status of these T-cell-specific regulatory elements affect Th1 lineage commitment in general and, in particular, during early Th1-cell differentiation, and whether IL-12 signaling regulates IFN-γ cytokine memory epigenetically. Such knowledge will provide insight into modulating Th1 responses in vaccine design, autoimmune diseases, and organ transplantation.

In this study, we identified a T-cell-specific methylation pattern at the promoter and CNS-1 of *IFNG* in ex vivo effector/memory Th1 cells. We further analyzed the methylation changes of these regions in $CD4^+$ T cells during their differentiation from naive T cells into IFN-γ-producing effector/memory Th1 cells. We show that the T-cell-specific hypomethylation at the *IFNG* promoter and CNS-1 could be verified by both in vitro differentiated Th1 cells and Ag-specific Th1 clones. In particular, the initiation of demethylation of the *IFNG* gene locus occurred before any cell cycle activity during Th1 differentiation. Progressive demethylation along the *IFNG* promoter and CNS-1 took place during the differentiation of naive T cells into early IFN-γ-producing Th1 cells and then to effector/memory Th1 cells. After termination of TCR stimulation, IL-12 signaling was required for early IFN-γ-producing Th1 cells to reinforce demethylation at the *IFNG* promoter and CNS-1. This led to the increased levels of hypomethylation at these two regions that resembled those in ex vivo IFN-γ effector/memory $CD4^+$ T lymphocytes, and the development of effector/memory Th1 cells. Therefore, our study provides an explanation of how epigenetic regulation establishes stable effector/memory during Th1-cell differentiation. Finally, the identification of demethylation of the *IFNG* promoter and CNS-1 shows clinical relevance in Th1-involved autoimmune diseases such as rheumatoid arthritis (RA).

Results

T-cell-specific hypomethylation of the *IFNG* locus in IFN-γ effector/memory $CD4^+$ T lymphocytes

To identify the T-cell-specific demethylation pattern of the *IFNG* gene locus, we first isolated ex vivo IFN-γ-producing and -nonproducing effector/memory Th-cell subsets from human peripheral blood using a cytokine capture assay (Fig. 1A). We then performed bisulfate PCR sequencing of seven CpG sites at CNS-1 (4.2 kb upstream from the transcriptional start site (TSS) of *IFNG*) and eight CpG sites at the promoter (Fig. 1B). At all CpGs of the promoter and CNS-1 regions, the IFN-γ^+ Th-cell subset showed hypomethylation, whereas the IFN-γ^- Th-cell subset showed hypermethylation (Fig. 1C). Because IFN-γ is also produced by $CD8^+$ T cells and NK cells, we further studied methylation status in different $CD8^+$ T and NK cells. Similarly to the $CD4^+$ Th-cell subset, naïve $CD8^+$ cells were hypermethylated, whereas effector and memory $CD8^+$ cells were hypomethylated (Supporting Information Fig. 1A). In contrast, NK cells displayed a differential methylation pattern with only a few CpGs being hypomethylated at the promoter (Supporting Information Fig. 1B). Notably, no differential methylations among all tested cell types were observed at a control region (2.4 kb upstream from the TSS of *IFNG* and adjacent to a DNase I hypersensitivity site) (Supporting Information Fig. 2A). Therefore, the distinct methylation patterns of $CD4^+$

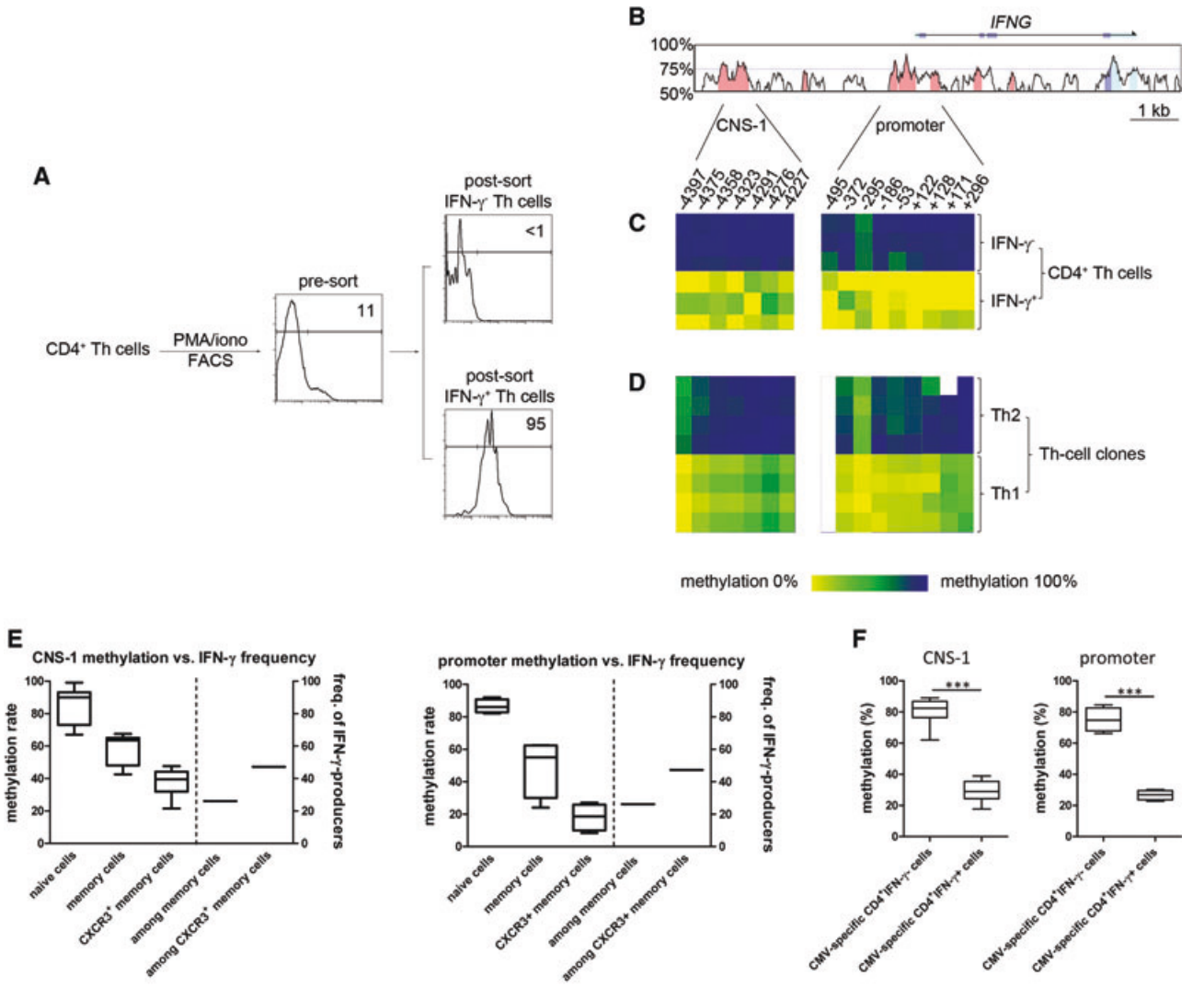


Figure 1. Inverse correlation between *IFNG* locus methylation and its expression in CD4$^+$ T cells. (A) Induction and isolation of ex vivo IFN-γ^- and IFN-γ^+ Th-cell subsets. CD4$^+$ T cells were stimulated with PMA and ionomycin, followed by an IFN-γ capture assay. The following Th cell subsets were identified: IFN-γ^- and IFN-γ^+. The data are representative of five separate experiments. (B) Alignment of 10 kb of the human and mouse *IFNG/Ifng* loci; DNA sequence identity >50% over at least 100 bp is shown in the histogram plot. Arrow, transcription direction; dark blue areas represent conserved exons; light blue areas represent conserved untranslated regions; reddish areas represent conserved noncoding sequences; individual CpGs at CNS-1 and the promoter are depicted in relation to the TSS. (C, D) The DNA methylation status of (C) three pooled DNA samples from each IFN-γ^- and IFN-γ^+ Th-cell subsets and (D) four Th1 and Th2 clones are shown in rows. (E) Inverse correlation between methylation levels of the promoter and CNS-1 with frequencies of IFN-γ-producing cells among memory and Th1 memory CD4$^+$ T cells. (F) T cell-specific hypomethylation at the *IFNG* promoter and CNS-1 in ex vivo CMV-specific CD4$^+$IFN-γ-producing effector/memory cells. DNA methylation levels were determined as previously described [11] (C and D) or average methylation levels of the promoter and CNS-1 by bisulfate pyrosequencing (E and F) are depicted with box plots with 5th and 95th percentile. $^{***}p < 0.0001$. Data shown are representative of two or three independent experiments.

and CD8$^+$ T cells and NK cells indicate that both the promoter and CNS-1 regions are important in the epigenetic regulation of selective T-cell differentiation. We next determined whether the T-cell-specific methylation pattern is heritable in Ag-specific Th clones and found that, in both of the promoter and CNS-1 regions, Th1 clones displayed a heritable hypomethylation pattern more similar to that of ex vivo IFN-γ^+ Th cells than to Th2 clones (Fig. 1D). We next assayed for the methylation status of naturally occurring effector/memory CD45RO$^+$ Th cells and CXCR3$^+$ Th1 cells in relation to their capacities to produce IFN-γ and found that, in both of the promoter and CNS-1 regions, the methylation status of these two memory cell types inversely correlated with frequencies of IFN-γ-producing cells (Fig. 1E). Finally, using an IFN-γ capture assay, we confirmed the T-cell-specific methylation pattern in ex vivo CMV-specific IFN-γ^+ Th cells (Fig. 1F). Taken together, we identified a T-cell-specific hypomethylation pattern of the *IFNG* gene locus in human effector/memory Th1 cells.

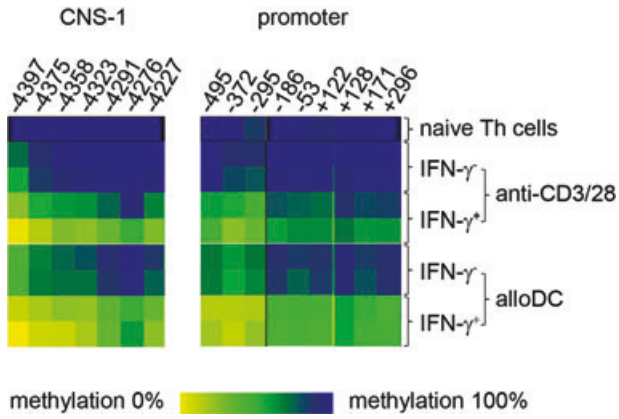


Figure 2. Th1 differentiation is accompanied by demethylation of CNS-1 and promoter of the *IFNG*. The methylation status of pooled (10 donors) naive Th cells and IFN-γ^- and IFN-γ^+ Th-cell subsets separated from 1 week Th1 cultures with anti-CD3/28 or alloDC stimulation from two independent experiments are shown in rows. DNA methylation levels were determined as described in Fig. 1C and D.

Demethylation dynamics of the *IFNG* gene locus during Th1 cell differentiation

The development of effector/memory status of $CD4^+$ T cells is reflected by the differentiation process from naïve Th cells to effector/memory IFN-γ-producing Th1 cells. With respect to this differentiation process, we next analyzed the methylation changes of the *IFNG* gene promoter and CNS-1 in naïve Th cells and in vitro-differentiated 1-week old Th1 cells separated according to the secretion of IFN-γ. In both the promoter and CNS-1 regions, naïve Th cells were hypermethylated, whereas IFN-γ^+ and IFN-γ^- Th1-cell subsets isolated from Th1 cultures activated with either plate-bound anti-CD3 and anti-CD28 or with alloDCs were hypomethylated and hypermethylated, respectively (Fig. 2). These results suggest that the T-cell-specific demethylation pattern of the *IFNG* promoter and CNS-1 is required for the induction of effector and/or memory Th1 cells during Th1-cell differentiation.

To explore the dynamic changes of demethylation of the *IFNG* gene triggered by early Th1 differentiation, we performed a 3-day time course analysis of the methylation changes in naïve Th cells activated by plate-bound anti-CD3 and anti-CD28 under Th1 conditions using bisulfate pyrosequencing technology. As shown in Figure 3A, in ex vivo-isolated naïve Th cells (0 h), CpGs methylation at CNS-1 was between 80 and 100%, and CpG methylation at the promoter was between 68 and 91%. Of note, 16 h after activation, the methylation level of CpG-4227 at CNS-1 was reduced from 80 to 70%. Similarly, after 19 h of activation, a 13% reduction in methylation (from 76 to 63%) was detected at CpG-186 at the promoter. In addition, between the 16 and 19 h time points, a 15% decrease in the methylation level of CpG-53 and a 35% decrease in the methylation level of CpG-186 (at the promoter) was also observed (Fig. 3A). However, methylation levels of the 2.4 kb upstream control region remained unchanged at these analyzed time points (Supporting Information Fig. 2B). Together, these results suggest that the dynamic changes in demethylation of the T-cell-specific epigenetic regulatory regions of the *IFNG* locus may be important in initiating the early Th1 differentiation.

To determine whether and at which time point the activated cells synthesize DNA, we combined time-course analysis with a BrdU incorporation assay. No $BrdU^+$ cells were detected at early time points (16, 19, or 22 h), while in contrast, increased numbers of $BrdU^+$ cells were observed later between 36 and 70 h (Fig. 3B), therefore suggesting that the initiation of demethylation of the *IFNG* gene implicates an active regulatory mechanism, independent of DNA replication.

We next investigated whether the methylation changes involve a cell division-dependent regulatory mechanism as measured by CFSE dilution of CFSE labeled naïve Th cells. Over time, primed cells expressed dynamic levels of *IFNG* and the Th1-lineage transcription factors *TBX21* and *RNX3* (unpublished observations), in agreement of recent results in mouse Th1 cells [7]. No cell divisions were observed 22 h after activation under Th1 conditions, and while some proliferation was detectable at 46 h, most cells required 70 h of activation to divide at least once (Fig. 3C). At these time points, drastic reduction (about 50%) in methylation was detectable at CpGs-4227 and -186 at CNS-1 and promoter, respectively (Fig. 3A), suggesting a potential contribution of proliferation to demethylation. To test this possibility, we analyzed methylation changes in cells sorted by cell division (Fig. 3D). In primed nondividing cells, the methylation levels of CpGs-4227, -4276, -4291, and -4323 at CNS-1 was reduced between 12 and 21% in comparison with ex vivo-isolated naive Th cells. However, when cells had undergone one or three cell divisions, the methylation levels of these CpGs were markedly reduced to half or even less than half of those of their previous cell division. Interestingly, a dynamic methylation pattern of CNS-1 was observed in subsequent cell divisions, similarly to that of the promoter and CNS-1 in bulk differentiating cultures. In addition, IFN-γ production according to distinct cell division was comparable with results shown in mouse Th1-cell cultures [16, 17] (Fig. 3E). Together, these results suggest an unrevealed complexity of demethylation of the *IFNG* locus during early Th1 differentiation, and this process is both proliferation-independent and -dependent.

Requirement of IL-12 signaling for the development of functional IFN-γ memory

To further decipher the impact of initial demethylation of the *IFNG* promoter and CNS-1 on the formation of IFN-γ memory, we investigated the mechanism by which naïve human $CD4^+$ T cells develop cytokine memory for IFN-γ after primary activation. Although it has been shown that *IFNG* mRNA is detectable as early as 2 h after activation [18], the first IFN-γ-producing effector cells were detectable only after 24 h (<0.5%), and their numbers increased over time (d2, 8 to 19%; d3, 30 to 40%) upon Th1 polarization by anti-CD3 and anti-CD28 stimulation (Fig. 4

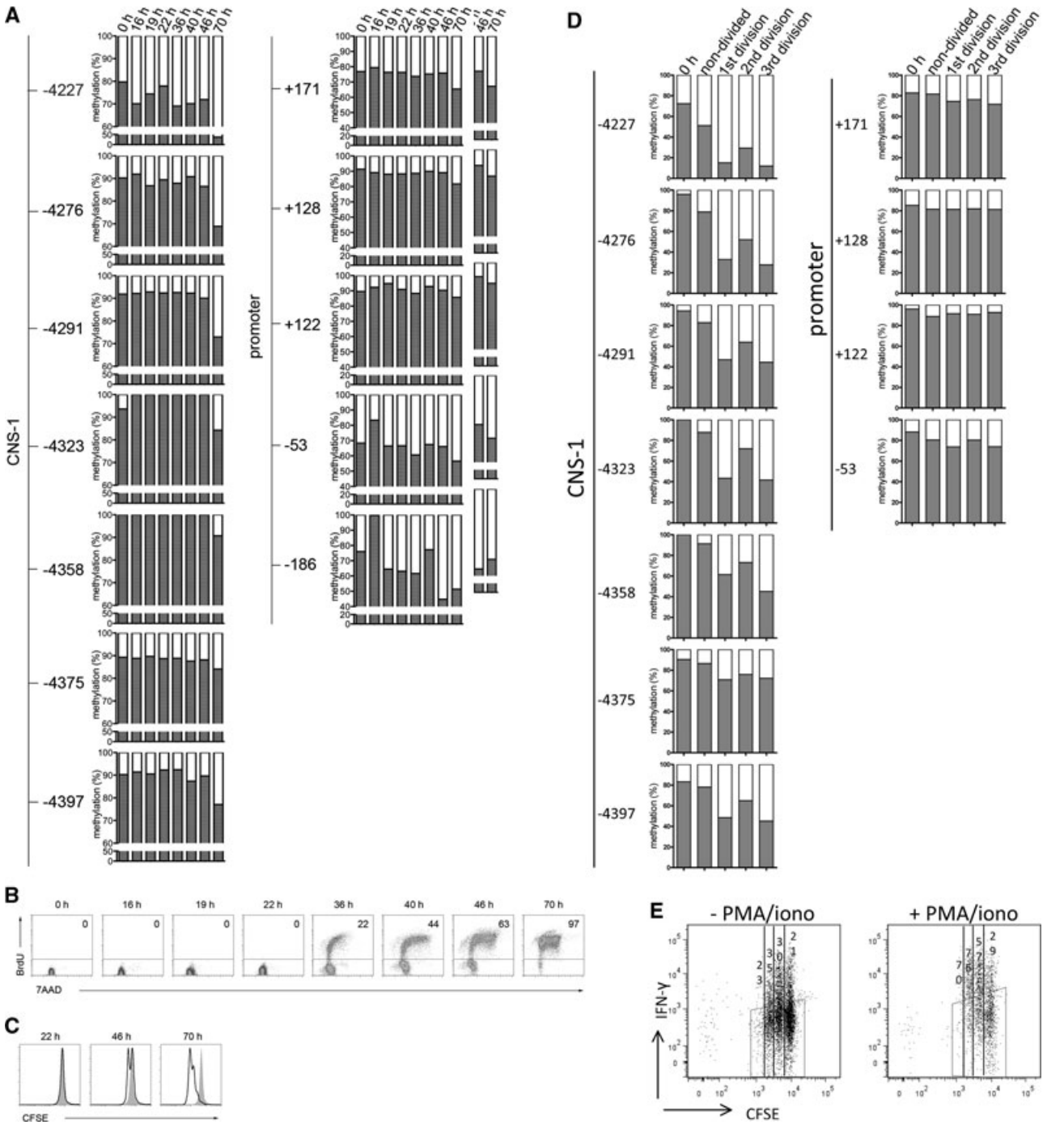


Figure 3. Dynamic demethylation at the *IFNG* locus during Th1 differentiation. (A) Time course analysis of the methylation status of individual CpGs in the CNS-1 and promoter regions during Th1 differentiation using bisulfate pyrosequencing. The data shown are from one experiment representative of two performed. Time course analysis of (B) DNA synthesis and (C) cell proliferation, and (D) dynamic methylation at the *IFNG* gene locus and (E) cytokine productions according to cell divisions during Th1-cell differentiation of the cells described in (A). The data shown are representative of three individual experiments.

and unpublished observations). To mimic Ag-specific TCR stimulation, we primed naive T cells for 2 days and separated early IFN-γ^+ and IFN-γ^- cells using the cytokine capture assay. IL-12, the major instructive signal in Th1 differentiation, is postulated to play a role in maintaining Th1-cell immunity in vivo in several experimental models [4, 19–21]. In addition, IL-12 has recently been shown to control *Ifng* reactivation after termination of antigen stimulation [7]. Therefore, the sorted d2-IFN-γ^+ and -IFN-γ^- cells were cultured in the presence or absence of IL-12 for 3 days and then restimulated with PMA and ionomycin (d7). In

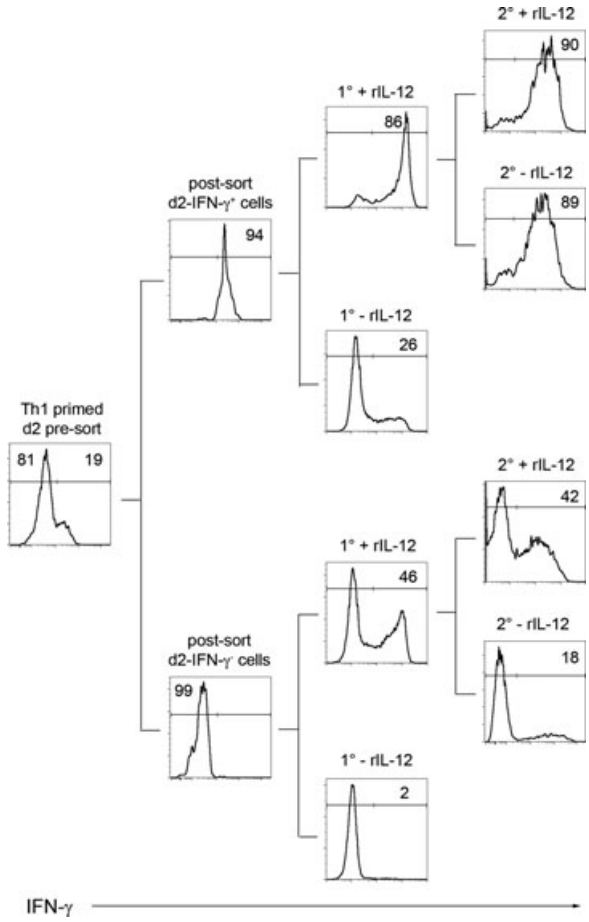


Figure 4. Th1 instruction signals and subsequent exposure to IL-12 are necessary and sufficient to establish long-term IFN-γ memory. Naïve T cells were primed in a Th1-cell culture with anti-CD3/28, followed by the IFN-γ capture assay. Presort and postsort histograms are shown. Isolated cells were cultured under neutral conditions with or without rIL-12 (1° ± rIL12) at day 0 and day 2. At day 7, IFN-γ reactivation was assessed by PMA and ionomycin stimulation. Cells receiving the initial IL-12 treatment were washed and further cultured under neutral conditions with or without rIL-12 (2° ± rIL12) and cytokine production was assessed as described above. The numbers indicate the percentage of IFN-γ-producing cells after PMA and ionomycin stimulation. The data shown are representative of at least three independent experiments.

response to the initial IL-12 stimulation (1° + rIL-12), d2-IFN-γ^+ cells were able to reactivate *IFNG* expression by 86%; d2-IFN-γ^- cells were induced to activate *IFNG* expression by 45%. In contrast, in the absence of IL-12 (1° – rIL-12), the majority of d2-IFN-γ^+ cells lost their ability to reproduce IFN-γ, whereas d2-IFN-γ^- cells remained negative for IFN-γ production. To determine whether the initial exposure to IL-12 is sufficient for d2-IFN-γ^+ cells to maintain long-term memory and, for d2-IFN-γ^- cells to maintain induced *IFNG* activation, cells at d7 were further cultured with or without IL-12 (2° ± rIL-12) and restimulated as described earlier. The d2-IFN-γ^+ cells that had received the initial IL-12 signal were able to reactivate *IFNG* expression by 90 and 89%, respectively, in response to further treatment with and without IL-12. Notably, IL-12-treated d2-IFN-γ^- cells behaved similarly in response to the second round of treatments (2° ± rIL-12) as did d2-IFN-γ^+ cells in response to the first round of treatments (1° ± rIL-12) (Fig. 4). Taken together, these data provide strong evidence that, after primary activation and termination of TCR stimulation, the original instructive IL-12 signal is required by early IFN-γ-producing Th1 cells to develop into stable effector/memory Th1 cells.

Epigenetic regulation of the *IFNG* for IFN-γ memory by IL-12 signaling in early IFN-γ^+ cells

Consistent with previous observations [6, 22], the responses of d2-IFN-γ^+ and -IFN-γ^- cells to IL-12 involved the tyrosine phosphorylation of STAT4 (unpublished observation). Both d2-IFN-γ^+ and -IFN-γ^- cells responded to 1° IL-12 treatment, whereas only d2-IFN-γ^+ cells promptly adopted a stable effector/memory fate. This observation led us to examine the demethylation status of the *IFNG* promoter and CNS-1 that was induced in early effector cells that received additional IL-12 stimulation. Therefore, we performed methylation analysis in d2-IFN-γ^+ and -IFN-γ^- cells, 1° IL-12-treated d2-IFN-γ^- cell-converted IFN-γ^+ and -remained IFN-γ^- cells, and 1° IL-12-treated d2-IFN-γ^+ cells that developed memory for *IFNG* reactivation.

As shown in Figure 5, at CNS-1, d2-IFN-γ^- cells were hypermethylated at all CpG sites and d2-IFN-γ^+ cells were generally hypomethylated. In particular, compared with naive cells (Fig. 3A), d2-IFN-γ^+ cells acquired 79% demethylation at CpG-4323 and 52, 40, 19, and 18% demethylation at CpG-4276, -4227, -4358, and -4291, respectively. Strikingly, in memory cells (IL-12-treated d2-IFN-γ^+) consisting of 86% IFN-γ-producers, the demethylation levels of these CpGs reached their maximal values (76 to 86%) compared with naive cells (Fig. 4). In addition, considerable levels of demethylation (62 and 21%) were also detected at another two CpGs (-4397 and -4375) in memory cells compared with the levels in naïve cells (Fig. 3A). Interestingly, 1° IL-12-treated d2-IFN-γ^- cell-converted IFN-γ^+ cells displayed a demethylation pattern similar to that of d2-IFN-γ^+ cells, whereas 1° IL-12-treated d2-IFN-γ^- cell-maintained IFN-γ^- cells were hypermethylated similar to d2-IFN-γ^- cells. Moreover, memory cells were able to maintain their demethylation status without further IL-12 signaling, whereas d2-IFN-γ^- cells required repetitive IL-12 signals to induce the demethylation of CNS-1 (unpublished observations). Moreover, the imprinting patterns of the promoter among analyzed cell types mirrored those of CNS-1. In particular, compared with naive cells, 60 and 61% demethylation was observed in 1° IL-12-treated d2-IFN-γ^+ cells at CpG-186 and -53, respectively (Fig. 5).

Thus, these results indicate that differential levels of demethylation of the *IFNG* promoter and CNS-1 in early IFN-γ^+ and IFN-γ^- cells underlie their differential fates. In particular, after termination of TCR stimulation, IL-12 signaling leads to reinforced levels of demethylation of the *IFNG* locus and ultimately the development of effector/memory Th1 cells from early effectors.

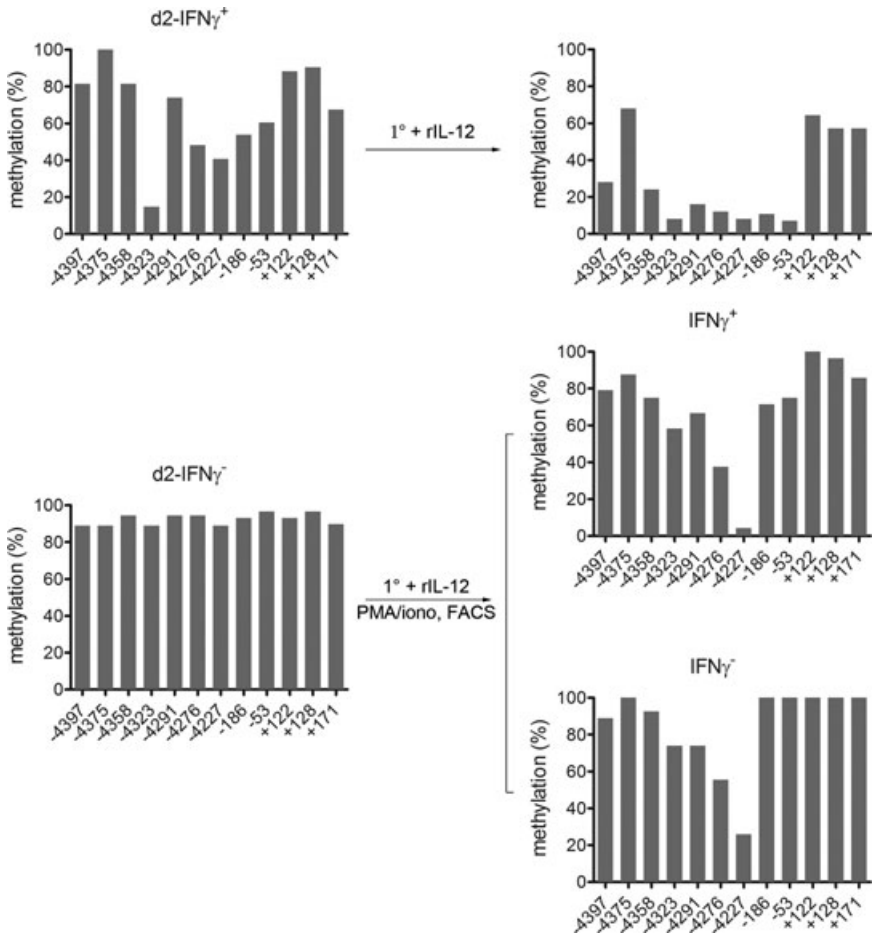


Figure 5. Distinct DNA methylation profiles of d2-IFN-γ^+ cells and -IFN-γ^- cells, 1° IL-12 treated d2-IFN-γ^- cell-converted IFN-γ^+ cells and -maintained IFN-γ^- cells, 1° IL-12-treated d2-IFN-γ^+ cells (as described in Fig. 4) at the *IFNG* locus. The data shown are representative of two independent experiments analyzed by bisulfate clone sequencing and pyrosequencing (not shown).

Methylation status of the *IFNG* promoter and CNS-1 in periphery memory T cells of patients with RA

Our results described earlier have clearly shown the association of demethylation of the *IFNG* promoter and CNS-1 and the development of IFN-γ memory Th1 cells. This promoted us to further study their clinical relevance in Th1-involved autoimmune diseases such as RA [23, 24]. Here, we analyzed the peripheral distribution of IFN-γ effector/memory Th1 cells by delineating the DNA methylation status of the *IFNG* promoter and CNS-1, among naïve and memory CD4$^+$ Th cell subsets between RA and healthy subjects. We also analyzed memory CD4$^+$ Th cells directly isolated from synovial fluid (SF) of inflamed joints of RA patients. Memory CD4$^+$ T cells consist of central memory and effector memory cell subsets, but IFN-γ are mainly produced by TEM cells [25]. Because naturally occurring CD45RO$^+$ and CXCR3$^+$ effector memory Th1 cells showed a similar pattern of epigenetic regulation which were inversely correlated with their frequencies of IFN-γ production, respectively (Fig. 1E), therefore in the following analyses we used total memory CD4$^+$ cells instead of further purifying memory cell subsets. In the naïve T-cell compartment, lower than 5% demethylation was observed in both the promoter and CNS-1, and there were no differences between RA patients and healthy controls. In contrast, in the memory CD4$^+$ Th-cell compartment of RA patients, 38% of such cells were demethylated, whereas the same cell type in healthy controls were 55% demethylated in the promoter. Similar to the promoter, in CNS-1, memory cells from RA patients were only 22% demethylated, whereas those from healthy controls were 39% demethylated. As expected, memory cells from SF were 85 and 67.5% demethylated in the promoter and CNS-1, respectively. Of note, the differences in both the promoter and CNS-1 between RA and healthy groups were significant (Fig. 6A). However, no differences in methylation levels were detected between purified IFN-γ-producers isolated from RA and healthy subjects (unpublished observations). In addition, the frequencies of IFN-γ-producing cells among effector/memory T cells of RA patients were significantly lower than those of healthy donors. The levels of DNA methylation correlated inversely with frequencies of IFN-γ-producing cells among memory T cells in both subject groups (Fig. 6B). Taken together, these results suggest that in RA patients reduced Th1 memory cells reside in the periphery blood, coincident with a selective recruitment of effector/memory Th1 cells to the site of inflammation.

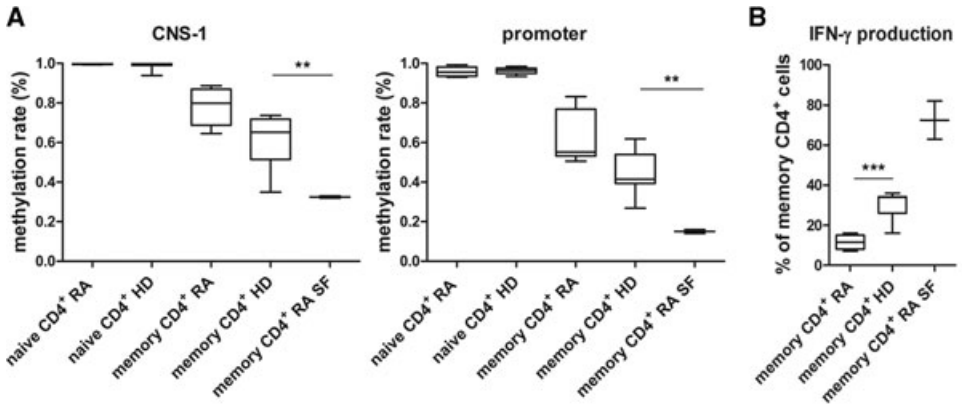


Figure 6. Coincidence of higher methylation levels of the *IFNG* promoter and CNS-1 in memory CD4$^+$ Th cells in the periphery and lower cytokine production in RA patients. (A) Methylation levels of the *IFNG* promoter and CNS-1 in peripheral memory CD4$^+$ Th cells of RA patients and healthy donors (HD) are shown. Ex vivo naive and/or memory CD4$^+$ T cells from RA patients (blood, $n = 8$; SF, $n = 2$) and HD ($n = 8$) were analyzed for methylation levels using bisulfate PCR sequencing and pyrosequencing approaches. (B) IFN-γ production in peripheral memory CD4$^+$ Th cells of RA patients and in HD are shown. Intracellular IFN-γ production was assessed after PMA and ionomycin stimulation. Data shown are from two independent cohorts of studies. Average methylation levels of the promoter and CNS-1 are depicted with box plots with 5th and 95th percentile. $^{**}p < 0.01$; $^{***}p < 0.0001$.

Discussion

We demonstrate here that early Th1-cell differentiation is accompanied by dynamic demethylation of CpGs at both the promoter and CNS-1 regions of the *IFNG* locus. Early epigenetic modification of the *IFNG* gene locus appears to be not only essential for early Th1 effector function, but that it is also indispensable for the establishment of stable functional Th1 cytokine memory. IL-12 signaling is required in this process after termination of TCR stimulation by strengthening the level of demethylation. Stable effector/memory Th1 cells are identified by hypomethylation of CpGs at the promoter and CNS-1 regions. In addition, analyzing methylation status of these two regions in memory Th-cell compartment can be used to evaluate the distribution and/or redistribution of effector/memory Th1 cells. Our data indicate an epigenetic mechanism of CpG demethylation that governs Th1-cell commitment.

Despite the identification of multiple distal regulatory elements regulating transcription of the murine *Ifng* gene, only the CNS-54, CNS-6 (correspond to human CNS-1), and CNS+18–20 showed murine Th1-specific demethylation [26]. However, a T-cell-specific role for CNS-54 in regulating transcription of *Ifng/IFNG* has not yet been shown, and regulation by CNS+20 is not specifically required by T cells [27]. Although conflicting data regarding a murine Th1-specific demethylation of the *Ifng* promoter have been reported [28, 29], the human *IFNG* proximal promoter confers Th1/Tc1 selective expression [14, 15]. Therefore, the promoter and CNS-1 of the human *IFNG* are to date the defined T-cell-specific regulatory elements. Indeed, in the present study the inverse correlation between DNA methylation and *IFNG* expression and the heritability of hypomethylation at the *IFNG* promoter and CNS-1 clearly demonstrate a T-cell-specific epigenetic regulatory role for these two regions. Interestingly, CpG-186 and -53 in the proximal promoter which are targets of general transcription factors such as CREB/ATF and AP-1, were about 70–90% methylated in naïve T cells, consistent with results obtained from murine naïve T cells [18]. This finding implies a limited degree of chromatin accessibility that allows rapid transcription, yet a low level, in the course of primary activation.

Two mechanisms by which DNA demethylates have been described to date. Passive demethylation occurs during DNA replication because of a failure to methylate CpGs in the newly synthesized strand if levels of DNA methyltransferase I (DnmtI) are insufficient. Active demethylation, on the other hand, takes place in the absence of DNA replication and cell division [30]. Both passive and active mechanisms have been observed during genome-wide demethylation after fertilization in mouse [31]. Active demethylation has been demonstrated in several systems [32–37]. Our observations that the demethylation of selective CpGs at the *IFNG* gene locus occurs in cell proliferation-independent and -dependent manner strongly suggest that the early demethylation of the *IFNG* gene during Th1-cell differentiation is regulated by both active and passive mechanisms. Active demethylation has been proposed to be mediated by the demethylases methyl CpG-binding domain (MBD) 2b and/or MBD 4 [38], through a DNA repair-like pathway [39], through activation-induced cytidine deaminase [40, 41], or through Tet enzymes that catalyze cytosine 5-hydroxymethylation [42, 43]. In this study, although the underlying mechanism remains unclear, both active and passive demethylation of the *IFNG* locus may positively regulate its expression during early Th1 differentiation.

By analyzing a 3-day time course of methylation changes in Th1-primed naive cells, we observed dynamic demethylation at several CpGs, such as CpG-4227, -186, and -53. Interestingly, recent studies also revealed that during the transcription dynamic DNA demethylation occurs in the promoter of genes such as the estrogen receptor α (ERα)-responsive gene *pS2* [44]. With respect

to cytokine gene regulation, a direct structure-function relationship between the spatial organization of the chromatin around the mouse *Ifng* gene and its transcriptional potential has been demonstrated [45]. Thus, the dynamic demethylation in early Th1 differentiation could be associated with the transcriptional activation of *IFNG*, thereby allowing a certain degree of flexibility in the regulation of gene expression in response to distinct stimuli and/or in conjunction with dynamic alterations of chromatin conformation.

We have previously shown that demethylation primarily drives *IFNG* expression in purified ex vivo $CD4^+$ T cells that do not originally produce IFN-γ [11]. However, the present data do not exclude the possibility that transcription factors (such as NFAT, PolII, T-bet, and STAT4, induced by TCR stimulation and instructive IL-12 signaling) affect *IFNG* activation by recruiting proteins that also modify the methylation status of *IFNG*. In this regard, a role for STAT4 in driving murine Th1 differentiation has been shown by STAT4-dependent changes in epigenetic histone modifications [46]. Importantly, the impact of CpG demethylation at the two Th1-specific regulatory regions in the *IFNG* locus on the development of memory cells was reflected by the differential demethylation status of d2-IFN-γ^+ and -IFN-γ^- cells and their different cell fates. Thus, when continuously receiving an IL-12 signal, d2-IFN-γ^+ cells with an initial level of CpG demethylation were able to maintain their effector function accompanied by the reinforced demethylation of these CpGs, whereas primed d2-IFN-γ^- cells needed repetitive IL-12 signaling to activate *IFNG*. Notably, the frequency of IFN-γ-producing cells in IL-12-cultured d2-IFN-γ^- cells remained consistent after further IL-12 treatment, but decreased in the absence of IL-12, suggesting that a fraction of the positive cells resemble d2-IFN-γ^+ cells. This is most likely because the demethylation pattern of the IFN-γ^+ cells sorted from 1° rIL12-cultured d2-IFN-γ^- cells resembles that of early d2-IFN-γ^+ cells, indicating that under certain circumstances, activated non-IFN-γ-producing Th cells may be geared towards effector-like or memory-like Th1 cells. In this regard, it has been shown that murine TCR-Tg IFN-γ^- cells can acquire the effector/memory phenotype in vivo through a delayed differentiation program [47].

We found that IL-12-treated d2-IFN-γ^+ cells exhibited a "poised" demethylated status of the *IFNG* gene similar to that of ex vivo memory Th1 cells. This demethylation imprinting of CpG sites at the proximal promoter and CNS-1 may provide direct access for the binding of lineage-transcription factors such as T-bet and STAT4, and for the binding of other general transcription factors, thereby serving as the basis for functional cytokine memory. DNA demethylation as the basis for such functional cytokine memory has been shown for murine *Il4* and human *IL2* [36, 48]. Moreover, our findings are in agreement with recent data from murine Th1 cells showing that enhanced IL-12 signaling through T-bet in the late phase of Th1 priming strongly correlates with the frequency of IFN-γ-producing cells in a later recall response [7]. Given the role of STAT4 in driving murine Th1 differentiation by STAT4-dependent changes in histone modifications [46, 49], it is attempting to speculate a IL-12-STAT4-dependent changes in DNA methylation of the *IFNG* gene may occur during the development effector/memory Th1 cells. Indeed, repetitive IL-12 stimulations lead to an inverse correlation between demethylation of the *IFNG* gene and *IFNG* expression in primed d2-IFN-γ^- cells, demonstrating that IL-12 is required not only in the differentiation of Th1 cells, but it is also required at multiple time points of Th1 differentiation to establish stable Th1 effector programs. Other cytokines such as IL-7 has been previously reported to enhance *IFNG* mRNA expression in activated T cells after 3 to 6 h of activation [50]. However, in our experimental system IL-7 stimulation alone or in combination with IL-15 did not lead to the transition from early effectors to effector/memory cells, therefore confirming that the effect of IL-12 stimulation cannot be replaced by IL-7 and/or IL-15.

Our findings of higher methylation levels of the *IFNG* promoter and CNS-1 in memory $CD4^+$ Th cells of peripheral blood in RA patients compared with those in SF indicate reduced memory Th1 cells in the periphery, coincident with recruitment of effector/memory Th1 cells to the site of inflammation. In support of this data, several studies have shown Th1-cell activity in the joint and reduced Th1 response in the periphery [23, 24]. Thus, analyzing DNA demethylation status of *IFNG* may serve as a novel and reliable method for evaluating the distribution and/or redistribution of effector/memory IFN-γ-producing cells in patients with Th1-involved autoimmune diseases.

In conclusion, TCR signaling and instructive Th1 signals (such as IL-12) induce dynamic demethylation of the *IFNG* locus, and the latter of which stabilizes heritable and stable *IFNG* hypomethylation and expression during the transition from effector to effector/memory Th1 cells. Our findings have major implications for researchers attempting to modulate Th1 responses in vaccine design, autoimmune diseases, and organ transplantation.

Materials and methods

Media and reagents

Cells were cultured in RPMI 1640 supplemented with 1% glutamax, 100 U/mL penicillin, 100 μg/mL streptomycin (Invitrogen Life Technologies), and 10% human AB serum (PAA). PMA (5 ng/mL; Sigma-Aldrich) and 1 μg/mL ionomycin (Sigma-Aldrich) were used for stimulation. Brefeldin A (5 μg/mL; Sigma-Aldrich) was used to block cytokine secretion. Recombinant (r) IL-7, rIL-15, rIL-12, and rIFN-γ, each at 10 ng/mL (R&D systems), were used to induce cell differentiation as indicated in the text.

Ex vivo cell purification

Buffy coats from anonymous healthy adult donors and patient materials were obtained with local ethical committee approvals and informed consents. PBMCs were isolated by

density gradient sedimentation using Ficoll-Hypaque (Sigma-Aldrich). $CD45RA^+CD31^+CD45RO^-$ naïve, $CXCR3^+CD45RO^+$ and $CD45RO^+$ memory $CD4^+$ Th-cell subsets were purified as described [51]. Monocytes were separated by the MACS positive selection of $CD14^+$ cells using CD14 microbeads (Miltenyi Biotec). The purity of the sorted populations was 95 to 99% as assessed by FACS.

Real-time quantitative PCR analysis

Total RNA from ex vivo or primed naïve $CD4^+$ Th cells at days 1, 2, and 3 was extracted using an RNeasy Mini Kit (Qiagen) and was reverse transcribed using TaqMan Reverse transcription reagents (Roche Applied Biosystems) according to the manufacturer's recommendations. cDNA was analyzed for the expression of *IFNG*, *TBX21*, *RUNX3* and the ubiquitin gene *UBCH5B* by real-time quantitative PCR analysis as previously described [11]. Primer sequence information is provided in Supporting Information Table 1.

In vitro generation of DCs

DCs were generated by culturing purified $CD14^+$ monocytes (1×10^5 cells/mL), in the presence of 50 ng/mL GM-CSF and 50 ng/mL IL-4 (both R&D Systems) for 5 days and 1 μg/mL LPS (Strathmann Biotech AG) for an additional 2 days. CD80, CD83, and CD86 (PE, FITC, and allophycocyanin, respectively; BD Biosciences) expression was used to monitor the maturation status of DCs. All of these markers were upregulated (higher than 80%) in all experiments.

In vitro generation of Th1 cells by alloDC or TCR stimulation

For Th1 differentiation, naive $CD4^+$ Th cells or CFSE-labeled (1 μM) naïve $CD4^+$ Th cells ($1–2 \times 10^6$/mL), as indicated, were primed with CFSE-labeled (5 μM; Molecular Probes) allogeneic monocyte-derived DCs (10:1) or plate-bound anti-CD3/CD28 (0.5 μg/mL/1 μg/mL; BD Biosciences) in the presence of 10 ng/mL rIFN-γ, rIL-12 (R&D Systems), and anti-IL-4 (10 μg/mL; BD Biosciences) for 2 or 3 days. Primed Th1 cells were analyzed for surface expressions such as CD45RO, IL18Rα (R&D Systems), CXCR3 (eBiosciences), and CD27, and intracellular cytokine productions such as IFN-γ and IL-2 using house conjugates.

IFN-γ capture assay

IFN-γ-producing Th cells from CMV-seropositive donors were isolated as previously described [11]. High frequency (>5%) of IFN-γ-producing Th cells were isolated with some modifications. Briefly, in vitro polarized Th1 cells, highly purified primary $CD4^+$ T lymphocytes or human antigen-specific Th1 clones were stimulated with PMA and ionomycin for 4 h, followed by the detection and isolation of IFN-γ-producing Th cells using a cytometric cytokine secretion assay (Miltenyi Biotec) and FACSDiva™ (BD Biosciences). To prevent nonspecific signaling, 10 or 1 μg/mL anti-IFN-γ was applied to the cell suspension before adding the catch reagent and the detection antibody, respectively. IFN-γ-producing Th cells and their counterparts were also directly isolated after 2 or 3 days Th1 priming with plate-bound anti-CD3/CD28.

Intracellular cytokine staining

For IFN-γ reactivation, naive cells were removed from the stimuli 2 days after priming and washed, or sorted into d2-IFN-γ^+ and -IFN-γ^- fractions. Subsequently, the cells were cultured for 1 to 3 weeks under one of the two following conditions: neutral (rIL-7/IL-15) or neutral plus rIL-12. The cell fractions were stimulated weekly with PMA and ionomycin for 4 h (the last 3 h with Brefeldin A), fixed, permeabilized, stained, and analyzed for intracellular IFN-γ. Alternatively, IFN-γ^+ and IFN-γ^- cells were sorted for methylation analysis.

Bisulfate-specific PCR (BSP) sequencing, clone sequencing, and pyrosequencing

Genomic DNA extraction and the bisulfate conversion were performed as described [11]. Regions of interests in the *IFNG* locus were BSP-amplified by primers designed by Primer3 software. PCR was performed and PCR product sequenced using the PCR primers and methylation levels calculated as described [11]. Alternatively, PCR product was cloned using a TOPO TA cloning kit (Invitrogen) and sequenced using vector-based primers from both directions (Eurofins MWG Operon or GATC biotech, Germany). In addition, BSP products were pyrosequenced by an ordered service (Varionostic GmbH, Germany). Briefly, 40 ng of bisulfite-converted DNA was used in a PCR using the following program: 95°C for 3 min, 50 cycles of 95°C for 35 s, 55°C for 35 s, and 72°C for 40 s, and a final extension at 72°C for 5 min. Methylation levels were analyzed using Pyro Q-CpG software. Primer sequences for the bisulfate PCR and sequencing are listed in Supporting Information Table 1.

Cell cycle analysis by BrdU incorporation

Cell cycle analysis was performed using an APC BrdU Flow kit (BD pharmingen) according to the manufacturer's instructions. Briefly, naive $CD4^+$ T cells were incubated with 10 μM BrdU under Th1 conditions. As a negative control, incubated cells were cultured in medium without activation. At indicated time points, cells were fixed, permeabilized, and stained with 7-AAD and anti-BrdU antibody. The stained cells were measured and the data were analyzed using FACSCalibur and Flowjo (Tree Star) software, respectively.

Statistics

Unpaired two-tailed Mann–Whitney tests were used for statistical analysis, with GraphPad Prism software.

Acknowledgements: We would like to acknowledge the assistances of Flow Cytometry Labs at the Berlin-Brandenburg Center for Regenerative Therapies and Deutsches Rheuma-Forschungszentrum Berlin. Special thanks to Dr. Mairi Mcgrath for critical proofreading of this manuscript. This work was in part supported by the BMBF (S-T-THERA 01GU0802 to A.T. and A.R.), the Berlin-Brandenburg Center for Regenerative Therapies (1. BCRT grant to J.D. and A.T.), and SFB 633 (A.T.).

Conflict of interest: The authors declare no financial or commercial conflict of interest.

References

1 **Ahmed, R. and Gray, D.**, Immunological memory and protective immunity: understanding their relation. *Science* 1996. **272**: 54–60.

2 **Zhu, J. and Paul, W. E.**, CD4 T cells: fates, functions, and faults. *Blood* 2008. **112**: 1557–1569.

3 **Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. and Coffman, R. L.**, Two types of murine helper T-cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* 1986. **136**: 2348–2357.

4 **Seder, R. A., Gazzinelli, R., Sher, A. and Paul, W. E.**, Interleukin 12 acts directly on CD4+ T cells to enhance priming for interferon gamma production and diminishes interleukin 4 inhibition of such priming. *Proc. Natl. Acad. Sci. USA* 1993. **90**: 10188–10192.

5 **Szabo, S. J., Kim, S. T., Costa, G. L., Zhang, X., Fathman, C. G. and Glimcher, L. H.**, A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 2000. **100**: 655–669.

6 **Thierfelder, W. E., van Deursen, J. M., Yamamoto, K., Tripp, R. A., Sarawar, S. R., Carson, R. T., Sangster, M. Y. et al.**, Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. *Nature* 1996. **382**: 171–174.

7 **Schulz, E. G., Mariani, L., Radbruch, A. and Hofer, T.**, Sequential polarization and imprinting of type 1 T helper lymphocytes by interferon-gamma and interleukin-12. *Immunity* 2009. **30**: 673–683.

8 **Lohning, M., Richter, A. and Radbruch, A.**, Cytokine memory of T helper lymphocytes. *Adv. Immunol.* 2002. **80**: 115–181.

9 **Wilson, C. B., Rowell, E. and Sekimata, M.**, Epigenetic control of T-helper-cell differentiation. *Nat. Rev. Immunol.* 2009. **9**: 91–105.

10 **Melvin, A. J., McGurn, M. E., Bort, S. J., Gibson, C. and Lewis, D. B.**, Hypomethylation of the interferon-gamma gene correlates with its expression by primary T-lineage cells. *Eur. J. Immunol.* 1995. **25**: 426–430.

11 **Dong, J., Ivascu, C., Chang, H. D., Wu, P., Angeli, R., Maggi, L., Eckhardt, F. et al.**, IL-10 is excluded from the functional cytokine memory of human CD4+ memory T lymphocytes. *J. Immunol.* 2007. **179**: 2389–2396.

12 **Mikovits, J. A., Young, H. A., Vertino, P., Issa, J. P., Pitha, P. M., Turcoski-Corrales, S. et al.**, Infection with human immunodeficiency virus type 1 upregulates DNA methyltransferase, resulting in de novo methylation of the gamma interferon (IFN-gamma) promoter and subsequent downregulation of IFN-gamma production. *Mol. Cell Biol.* 1998. **18**: 5166–5177.

13 **Lee, D. U., Avni, O., Chen, L. and Rao, A.**, A distal enhancer in the interferon-gamma (IFN-gamma) locus revealed by genome sequence comparison. *J. Biol. Chem.* 2004. **279**: 4802–4810.

14 **Penix, L. A., Sweetser, M. T., Weaver, W. M., Hoeffler, J. P., Kerppola, T. K. and Wilson, C. B.**, The proximal regulatory element of the interferon-gamma promoter mediates selective expression in T cells. *J. Biol. Chem.* 1996. **271**: 31964–31972.

15 **Soutto, M., Zhang, F., Enerson, B., Tong, Y., Boothby, M. and Aune, T. M.**, A minimal IFN-gamma promoter confers Th1 selective expression. *J. Immunol.* 2002. **169**: 4205–4212.

16 **Bird, J. J., Brown, D. R., Mullen, A. C., Moskowitz, N. H., Mahowald, M. A., Sider, J. R., Gajewski, T. F. et al.**, Helper T-cell differentiation is controlled by the cell cycle. *Immunity* 1998. **9**: 229–237.

17 **Richter, A., Lohning, M. and Radbruch, A.**, Instruction for cytokine expression in T helper lymphocytes in relation to proliferation and cell cycle progression. *J. Exp. Med.* 1999. **190**: 1439–1450.

18 **Letimier, F. A., Passini, N., Gasparian, S., Bianchi, E. and Rogge, L.**, Chromatin remodeling by the SWI/SNF-like BAF complex and STAT4 activation synergistically induce IL-12Rbeta2 expression during human Th1 cell differentiation. *EMBO J.* 2007. **26**: 1292–1302.

19 **Stobie, L., Gurunathan, S., Prussin, C., Sacks, D. L., Glaichenhaus, N., Wu, C. Y. and Seder, R. A.**, The role of antigen and IL-12 in sustaining Th1 memory cells in vivo: IL-12 is required to maintain memory/effector Th1 cells sufficient to mediate protection to an infectious parasite challenge. *Proc. Natl. Acad. Sci. USA* 2000. **97**: 8427–8432.

20 **Park, A. Y., Hondowicz, B. D. and Scott, P.**, IL-12 is required to maintain a Th1 response during Leishmania major infection. *J. Immunol.* 2000. **165**: 896–902.

21 **Yap, G., Pesin, M. and Sher, A.**, Cutting edge: IL-12 is required for the maintenance of IFN-gamma production in T cells mediating chronic resistance to the intracellular pathogen, Toxoplasma gondii. *J Immunol* 2000. **165**: 628–631.

22 **Jacobson, N. G., Szabo, S. J., Weber-Nordt, R. M., Zhong, Z., Schreiber, R. D., Darnell, J. E., Jr. and Murphy, K. M.**, Interleukin 12 signaling in T helper type 1 (Th1) cells involves tyrosine phosphorylation of signal transducer and activator of transcription (Stat)3 and Stat4. *J. Exp. Med.* 1995. **181**: 1755–1762.

23 **Janossy, G., Panayi, G., Duke, O., Bofill, M., Poulter, L. W. and Goldstein, G.**, Rheumatoid arthritis: a disease of T-lymphocyte/macrophage immunoregulation. *Lancet* 1981. **2**: 839–842.

24 **Firestein, G. S.**, Immunologic mechanisms in the pathogenesis of rheumatoid arthritis. *J. Clin. Rheumatol.* 2005. **11**: S39–44.

25 **Sallusto, F., Geginat, J. and Lanzavecchia, A.**, Central memory and effector memory T-cell subsets: function, generation, and maintenance. *Annu. Rev. Immunol.* 2004. **22**: 745–763.

26 **Schoenborn, J. R., Dorschner, M. O., Sekimata, M., Santer, D. M., Shnyreva, M., Fitzpatrick, D. R., Stamatoyannopoulos, J. A. and Wilson, C. B.**, Comprehensive epigenetic profiling identifies multiple distal regulatory elements directing transcription of the gene encoding interferon-gamma. *Nat. Immunol.* 2007. **8**: 732–742.

27 **Collins, P. L., Henderson, M. A. and Aune, T. M.**, Diverse functions of distal regulatory elements at the IFNG locus. *J. Immunol.* 2012. **188**: 1726–1733.

28 **Winders, B. R., Schwartz, R. H. and Bruniquel, D.**, A distinct region of the murine IFN-gamma promoter is hypomethylated from early T-cell

development through mature naive and Th1 cell differentiation, but is hypermethylated in Th2 cells. *J. Immunol.* 2004. **173**: 7377–7384.

29 **Jones, B. and Chen, J.**, Inhibition of IFN-gamma transcription by site-specific methylation during T helper cell development. *EMBO J.* 2006. **25**: 2443–2452.

30 **Bird, A.**, DNA methylation patterns and epigenetic memory. *Genes Dev.* 2002. **16**: 6–21.

31 **Gjerset, R. A. and Martin, D. W., Jr.,** Presence of a DNA demethylating activity in the nucleus of murine erythroleukemic cells. *J. Biol. Chem.* 1982. **257**: 8581–8583.

32 **Oswald, J., Engemann, S., Lane, N., Mayer, W., Olek, A., Fundele, R., Dean, W., Reik, W. and Walter, J.**, Active demethylation of the paternal genome in the mouse zygote. *Curr. Biol.* 2000. **10**: 475–478.

33 **Chen, W. G., Chang, Q., Lin, Y., Meissner, A., West, A. E., Griffith, E. C., Jaenisch, R. and Greenberg, M. E.**, Derepression of BDNF transcription involves calcium-dependent phosphorylation of MeCP2. *Science* 2003. **302**: 885–889.

34 **Martinowich, K., Hattori, D., Wu, H., Fouse, S., He, F., Hu, Y., Fan, G. and Sun, Y. E.**, DNA methylation-related chromatin remodeling in activity-dependent BDNF gene regulation. *Science* 2003. **302**: 890–893.

35 **Bruniquel, D. and Schwartz, R. H.**, Selective, stable demethylation of the interleukin-2 gene enhances transcription by an active process. *Nat. Immunol.* 2003. **4**: 235–240.

36 **Murayama, A., Sakura, K., Nakama, M., Yasuzawa-Tanaka, K., Fujita, E., Tateishi, Y., Wang, Y. et al.**, A specific CpG site demethylation in the human interleukin 2 gene promoter is an epigenetic memory. *EMBO J.* 2006. **25**: 1081–1092.

37 **Klug, M., Heinz, S., Gebhard, C., Schwarzfischer, L., Krause, S. W., Andreesen, R. and Rehli, M.**, Active DNA demethylation in human post-mitotic cells correlates with activating histone modifications, but not transcription levels. *Genome Biol.* 2010. **11**: R63.

38 **Kim, M. S., Kondo, T., Takada, I., Youn, M. Y., Yamamoto, Y., Takahashi, S., Matsumoto, T. et al.**, DNA demethylation in hormone-induced transcriptional derepression. *Nature* 2009. **461**: 1007–1012.

39 **Kress, C., Thomassin, H. and Grange, T.**, Active cytosine demethylation triggered by a nuclear receptor involves DNA strand breaks. *Proc. Natl. Acad. Sci. USA* 2006. **103**: 11112–11117.

40 **Popp, C., Dean, W., Feng, S., Cokus, S. J., Andrews, S., Pellegrini, M., Jacobsen, S. E. and Reik, W.**, Genome-wide erasure of DNA methylation in mouse primordial germ cells is affected by AID deficiency. *Nature* 2010. **463**: 1101–1105.

41 **Bhutani, N., Brady, J. J., Damian, M., Sacco, A., Corbel, S. Y. and Blau, H. M.**, Reprogramming towards pluripotency requires AID-dependent DNA demethylation. *Nature* 2010. **463**: 1042–1047.

42 **Tahiliani, M., Koh, K. P., Shen, Y., Pastor, W. A., Bandukwala, H., Brudno, Y., Agarwal, S. et al.**, Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science* 2009. **324**: 930–935.

43 **Ito, S., D'Alessio, A. C., Taranova, O. V., Hong, K., Sowers, L. C. and Zhang, Y.**, Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. *Nature* 2010. **466**: 1129–1133.

44 **Metivier, R., Gallais, R., Tiffoche, C., Le Peron, C., Jurkowska, R. Z., Carmouche, R. P., Ibberson, D. et al.**, Cyclical DNA methylation of a transcriptionally active promoter. *Nature* 2008. **452**: 45–50.

45 **Eivazova, E. R. and Aune, T. M.**, Dynamic alterations in the conformation of the Ifng gene region during T helper cell differentiation. *Proc. Natl. Acad. Sci. USA* 2004. **101**: 251–256.

46 **Wei, L., Vahedi, G., Sun, H. W., Watford, W. T., Takatori, H., Ramos, H. L., Takahashi, H. et al.**, Discrete roles of STAT4 and STAT6 transcription factors in tuning epigenetic modifications and transcription during T helper cell differentiation. *Immunity* 2010. **32**: 840–851.

47 **Wu, C. Y., Kirman, J. R., Rotte, M. J., Davey, D. F., Perfetto, S. P., Rhee, E. G., Freidag, B. L. et al.**, Distinct lineages of T(H)1 cells have differential capacities for memory cell generation in vivo. *Nat. Immunol.* 2002. **3**: 852–858.

48 **Tykocinski, L. O., Hajkova, P., Chang, H. D., Stamm, T., Sozeri, O., Lohning, M., Hu-Li, J. et al.**, A critical control element for interleukin-4 memory expression in T helper lymphocytes. *J. Biol. Chem.* 2005. **280**: 28177–28185.

49 **Morinobu, A., Kanno, Y. and O'Shea, J. J.**, Discrete roles for histone acetylation in human T helper 1 cell-specific gene expression. *J. Biol. Chem.* 2004. **279**: 40640–40646.

50 **Borger, P., Kauffman, H. F., Postma, D. S. and Vellenga, E.**, Interleukin-4 gene expression in activated human T lymphocytes is regulated by the cyclic adenosine monophosphate-dependent signaling pathway. *Blood* 1996. **87**: 691–698.

51 **Kimmig, S., Przybylski, G. K., Schmidt, C. A., Laurisch, K., Mowes, B., Radbruch, A. and Thiel, A.**, Two subsets of naive T helper cells with distinct T-cell receptor excision circle content in human adult peripheral blood. *J. Exp. Med.* 2002. **195**: 789–794.

Abbreviations: **BSP**: bisulfate-specific PCR · **HD**: healthy donor · **RA**: rheumatoid arthritis · **SF**: synovial fluid

Full correspondence: Dr. Jun Dong, Cell Biology Group, German Rheumatism Research Centre Berlin 1, 10117, Berlin, Germany
Fax: +49-30-28460603
e-mail: dong@drfz.de

Additional correspondence: Prof. Dr. Andreas Thiel, Regenerative Immunology and Aging, Berlin-Brandenburg Center for Regenerative Therapies, Charité, Campus Virchow-Klinikum Augustenburger Platz 1, 13353, Berlin, Germany
Fax: +49-30-450539955
e-mail: andreas.thiel@charite.de

Received: 26/7/2012
Revised: 19/11/2012
Accepted: 12/12/2012
Accepted article online: 19/12/2012

3.5 Twist1 reguliert pro-inflammatorische Funktionen von chronisch aktivierten Th1-Zellen

Niesner U, Albrecht I, Janke M, Doebis C, Loddenkemper C, Lexberg MH, Eulenburg K, Kreher S, Koeck J, Baumgrass R, Bonhagen K, Kamradt T, Enghard P, Humrich JY, Rutz S, Schulze-Topphoff U, Aktas O, Bartfeld S, Radbruch H, Hegazy AN, Löhning M, Baumgart DC, Duchmann R, Rudwaleit M, Häupl T, Gitelman I, Krenn V, Gruen J, Sieper J, Zeitz M, Wiedenmann B, Zipp F, Hamann A, Janitz M, Scheffold A, Burmester GR, **Chang HD**, Radbruch A. 2008. Autoregulation of Th1-mediated inflammation by twist1. *J Exp Med*. 205(8):1889-901.

Th-Zellen spielen eine zentrale Rolle bei der Initiierung und der Aufrechterhaltung von chronischen Entzündungen. Sie sind aber auch essentiell für protektive Immunität. Therapien die gegen Th-Zellen gerichtet waren, wie z.B. die Eliminierung aller Th-Zellen mittels anti-CD4-Antikörper, hatten begrenzten klinischen Erfolg, wahrscheinlich wegen mangelnder Selektivität für pathogene Th-Zellen.

Ziel dieser Arbeit war es Gene zu identifizieren, die in pathogenen, entzündungsfördernden Th-Zellen exprimiert werden und die sich als mögliche therapeutische Zielstrukturen nutzen lassen, um solche Zellen selektiv zu eliminieren. In der Annahme, dass Th-Zellen, die aktiv eine chronische Entzündung antreiben, ständig über ihr spezifisches Antigen aktiviert werden, haben wir Th-Zellen unter verschiedenen polarisierenden Bedingungen mehrfach *in vitro* aktiviert. Um Gene zu identifizieren, die selektiv in chronisch-aktivierten Th-Zellen angeschaltet werden, haben wir die Transkriptome von mehrfach *in vitro* aktivierten Th-Zellen mit denen von nur einmal aktivierten Th-Zellen verglichen. In mehrfach aktivierten Th1-Zellen wurde das Gen *Twist1* hoch exprimiert. Die Twist1-Expression in Th1-Zellen nahm mit jeder Reaktivierung-zu. Wir konnten zeigen, dass die initiale Twist1-Induktion durch IL-12/STAT4 und einem bisher noch nicht identifizierten Signal von B Zellen geschieht. Eine ektopische Überexpression von Twist1 in Th-Zellen resultierte in einer reduzierten Expression der pro-inflammatorischen Zytokine IFNγ und TNFα. Entsprechend haben Th1-Zellen, in denen wir die Twist1-Expression mittels spezifischen short-hairpin (sh)RNAs drastisch reduziert haben, im Model der ovalbumin-induzierten Arthritis eine verstärkte Entzündung ausgelöst. Somit konnten wir zeigen, dass Twist1 eine intrinsische regulatorische Rolle bei der Limitierung der Th-Zell-vermittelten Entzündung spielt.

Wir konnten auch zeigen, dass Twist1 in Th-Zellen, die wir direkt aus dem entzündeten Gewebe von Patienten mit unterschiedlichsten chronisch-entzündlichen Krankheiten isoliert haben, hoch exprimiert ist. Diese Patienten hatten trotz Immunsuppression eine aktive Entzündung. Dies bedeutet zum einen, dass trotz Immunsuppression Twist1-exprimierende Th-Zellen im entzündeten Gewebe persistieren und zum anderen, dass Twist1 neben einer regulatorischen Rolle anscheinend noch weitere Funktionen in chronisch aktivierten Th-Zellen ausübt.

Published July 28, 2008

Autoregulation of Th1-mediated inflammation by *twist1*

Uwe Niesner,[1] Inka Albrecht,[1] Marko Janke,[1] Cornelia Doebis,[2] Christoph Loddenkemper,[4] Maria H. Lexberg,[1] Katharina Eulenburg,[2] Stephan Kreher,[1] Juliana Koeck,[1] Ria Baumgrass,[1] Kerstin Bonhagen,[6] Thomas Kamradt,[6] Philipp Enghard,[1] Jens Y. Humrich,[1] Sascha Rutz,[1] Ulf Schulze-Topphoff,[7] Orhan Aktas,[7] Sina Bartfeld,[1] Helena Radbruch,[7] Ahmed N. Hegazy,[1] Max Löhning,[2] Daniel C. Baumgart,[8] Rainer Duchmann,[5] Martin Rudwaleit,[5] Thomas Häupl,[2] Inna Gitelman,[9] Veit Krenn,[3] Joachim Gruen,[1] Jochen Sieper,[2] Martin Zeitz,[5] Bertram Wiedenmann,[8] Frauke Zipp,[7] Alf Hamann,[2] Michal Janitz,[10] Alexander Scheffold,[1] Gerd R. Burmester,[2] Hyun D. Chang,[1] and Andreas Radbruch[1]

[1]German Rheumatism Research Center Berlin, 10117 Berlin, Germany
[2]Department of Rheumatology and Clinical Immunology, [3]Institute of Pathology, Charité-University Medicine Berlin, 10117 Berlin, Germany
[4]Department of Pathology/RCIS, [5]Medical Clinic I (Gastroenterology, Rheumatology, Infectiology), Charité-University Medicine Berlin, Campus Benjamin Franklin, 12200 Berlin, Germany
[6]Institute of Immunology, Friedrich Schiller University Jena, Medical School, 07740 Jena, Germany
[7]Cecilie Vogt Clinic for Neurology in the HKBB, Charité–University Medicine Berlin, and Max Delbrück Center for Molecular Medicine, 10117 Berlin, Germany
[8]Department of Medicine, Division of Hepatology and Gastroenterology, Charité-University Medicine Berlin, Humboldt University of Berlin, 13344 Berlin, Germany
[9]Department of Virology and Developmental Genetics, Ben Gurion University of the Negev, Beer Sheva 84105, Israel
[10]Max Planck Institute for Molecular Genetics, Department of Vertebrate Genomics, 14195 Berlin, Germany

The basic helix-loop-helix transcriptional repressor *twist1*, as an antagonist of nuclear factor κB (NF-κB)–dependent cytokine expression, is involved in the regulation of inflammation-induced immunopathology. We show that *twist1* is expressed by activated T helper (Th) 1 effector memory (EM) cells. Induction of *twist1* in Th cells depended on NF-κB, nuclear factor of activated T cells (NFAT), and interleukin (IL)-12 signaling via signal transducer and activator of transcription (STAT) 4. Expression of *twist1* was transient after T cell receptor engagement, and increased upon repeated stimulation of Th1 cells. Imprinting for enhanced *twist1* expression was characteristic of repeatedly restimulated EM Th cells, and thus of the pathogenic memory Th cells characteristic of chronic inflammation. Th lymphocytes from the inflamed joint or gut tissue of patients with rheumatic diseases, Crohn's disease or ulcerative colitis expressed high levels of *twist1*. Expression of *twist1* in Th1 lymphocytes limited the expression of the cytokines interferon-γ, IL-2, and tumor necrosis factor-α, and ameliorated Th1-mediated immunopathology in delayed-type hypersensitivity and antigen-induced arthritis.

CORRESPONDENCE
Andreas Radbruch:
radbruch@drfz.de

Abbreviations used: CD, Crohn's disease; ChIP, chromatin immunoprecipitation; CM, central memory; DTH, delayed-type hypersensitivity; EM, effector memory; shRNA, small hairpin RNA; UC, ulcerative colitis.

Twist1 and the closely related *twist2* (also known as *dermo1*) genes encode basic helix-loop-helix transcription factors involved in the formation of mesoderm in *Drosophila melanogaster* (1), cranial neural tube and limb morphogenesis in mice (2), metastasis of tumor cells (3), control of apoptosis, and expression of cytokine genes in inflammation (4, 5). Mice deficient for *twist2* or haploinsufficient for both *twist1* and *twist2* succumb to severe systemic

U. Niesner and I. Albrecht contributed equally to this paper.
The online version of this article contains supplemental material.

The Rockefeller University Press $30.00
J. Exp. Med. Vol. 205 No. 8 1889-1901
www.jem.org/cgi/doi/10.1084/jem.20072468

Published July 28, 2008

inflammation, demonstrating the central role of Twist proteins in the regulation of inflammation. *Twist1* and *twist2* are expressed by fibroblasts and macrophages, and expression is promoted by TNF-α and type I IFNs (4, 5). The involvement of Twist expression by fibroblasts and macrophages in the control of inflammation is probable, but has not been investigated in detail so far.

T helper 1 (Th1) lymphocytes are potent inducers of inflammation. This has been demonstrated by adoptive transfer of Th1 lymphocytes in murine models of Th1-associated inflammatory diseases such as diabetes (6), inflammatory bowel disease (7), and rheumatoid arthritis (8). In these models, the induction of inflammation in a particular tissue by Th1 lymphocytes is dependent on restimulation by the cognate antigen in that tissue. Induction of inflammation by Th1 cells is mediated by expression of the proinflammatory cytokines TNF-α and IFN-γ, the latter being a hallmark of Th1 differentiation. IFN-γ also induces expression of the chemokine receptor CXCR3 and its ligands CXCL9, -10, and -11, attracting Th1 cells specifically to inflamed tissue (9). Th cells with the capacity to recall IFN-γ expression, i.e., Th1 memory cells, are detectable in chronically inflamed tissue (10, 11). The role of Th1 cells in the development and maintenance of chronic inflammation is less clear. Anti–IFN-γ therapy has been shown to be beneficial in various Th1-associated inflammatory diseases (12, 13). However, a regulatory role for IFN-γ has also been demonstrated, based on the induction of inducible nitric oxide synthase (14) and of IL-12 in antigen-presenting cells, in turn increasing the IL-10 synthesis of Th1 cells (15). Furthermore, IFN-γ inhibits the differentiation of naive Th cells into proinflammatory Th17 cells (16, 17).

In this study, we demonstrate specific autoregulation of Th1 cells by the transcription factor *twist1*. Expression of *twist1* in Th cells is induced by IL-12/STAT4, NF-κB, and NFAT, and thus is specific for Th1 cells. Th1 effector memory (EM) cells (18) show increased transient reexpression of *twist1* upon T cell receptor engagement. *Twist1* reduces the functionality of Th1 cells by attenuating expression of IL-2, TNF-α, and IFN-γ. Th cells isolated from chronically inflamed gut tissue of patients with ulcerative colitis (UC) or Crohn's disease (CD) and synovial fluid of patients with spondyloarthropathies or rheumatoid arthritis are imprinted to express high levels of *twist1*, which indicates a history of repeated restimulation and an involvement in the pathogenesis of the disease. In murine models of acute and chronic inflammation, i.e., delayed-type hypersensitivity (DTH) and antigen-induced arthritis, expression of *twist1* by Th1 cells regulates Th1-mediated inflammation.

RESULTS

Twist1 is transiently expressed in repeatedly activated Th1 cells

To define transcriptional changes during Th1 memory cell differentiation, we compared the global gene expression of murine Th1 cells, which were activated with antigen once or repeatedly at 6-d intervals. Naive $CD4^+CD62L^{hi}$ T lymphocytes expressing the transgenic DO11.10 TCR specific for OVA were activated in vitro with splenic APCs and the cognate peptide $OVA_{327-339}$ under conditions that induce functional differentiation into Th1 cells, i.e., addition of IL-12 and blocking antibodies specific for IL-4. The transcriptional profiles of once- and four-time–stimulated Th1 cells were compared using high-density DNA microarrays. Among the 17 genes differentially expressed by a factor of two or more was *twist1*. Expression of *twist1* was up-regulated 38-fold in four-time– versus once-stimulated Th1 cells (Table S1, available at http://www.jem.org/cgi/content/full/jem.20072468/DC1).

Twist1 expression in Th1, but not Th2 or Th17, cells was confirmed by real-time PCR (Fig. 1). *Twist1* mRNA expression in resting Th1 cells correlated with their age in vitro and the number of restimulations they had experienced. Expression was further enhanced 3 h after polyclonal stimulation either with PMA and the Ca^{2+} ionophore ionomycin, or with CD3- and CD28-specific antibodies (Fig. 1 A). When determined 3 h after restimulation with anti-CD3/-CD28 antibodies, expression of *twist1* was close to the detection limit in naive Th cells. Expression increased ∼15-fold during the first Th1-polarizing stimulation, another 10-fold during the second stimulation, and another 3-fold during the third stimulation, reaching the maximum level after the fourth stimulation and remaining stable thereafter (Fig. 1 A). With PMA/ionomycin restimulation, maximum levels of *twist1* mRNA were reached after two rounds of stimulation. In Th1 cells, upon anti-CD3/-CD28 restimulation, *twist1* mRNA expression was up-regulated within the first hour, reaching maximum levels after 3 h, and decreasing again thereafter (Fig. 1 B). Expression of *twist1* mRNA was also detectable in restimulated Th2 cells, but its level remained 30-fold lower than in Th1 cells. In Th17 cells, the level of *twist1* mRNA expression was even lower than in Th2 cells (Fig. 1 C).

Twist1 protein was detectable in 6-d-old restimulated Th1 cells. In 24-d-old Th1 cells, expression was enhanced. In resting Th1 cells and in reactivated or resting Th2 cells, Twist1 was not detectable by immunoblotting (Fig. 1 D). In agreement with the expression of *twist1* mRNA, Twist1 protein expression peaked 3 h after reactivation, and then ceased, with levels still detectable 48 h after restimulation (Fig. 1 E), but not at 6 d after restimulation.

Twist2 was not expressed in the Th1 and Th2 cells analyzed here, as determined by real-time PCR (unpublished data).

Control of *twist1* expression in Th1 lymphocytes

The specific expression of *twist1* by activated Th1 cells suggests that Th1-polarizing or Th1-specific signals are required for the induction of *twist1* expression in Th cells. Indeed, IL-12/STAT4 signaling induced *twist1* expression in a dose-dependent fashion (Fig. 2 A). IFN-γ and STAT1 signaling did not induce *twist1* expression in the absence of IL-12 (Fig. 2 A). T-bet, a T box transcription factor sufficient to induce Th1 differentiation (19), is not required for induction of expression of *twist1*; induction *of twist1* by IL-12 was comparable in T-bet–deficient

Published July 28, 2008

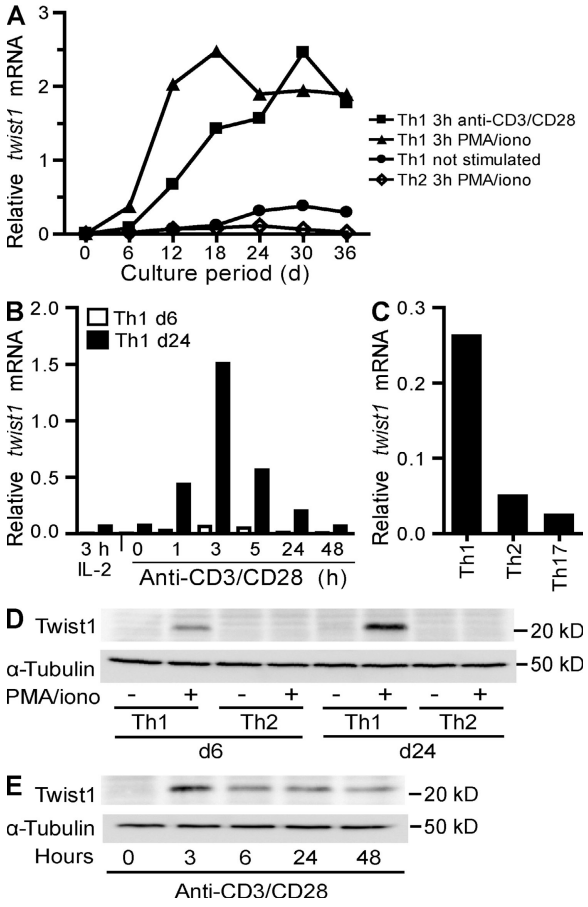


Figure 1. *Twist1* expression is induced in Th1, but not Th2 or Th17 cells. $CD4^+CD62L^{hi}$ OVA-specific T lymphocytes were stimulated in vitro under Th1 or Th2, or under Th17-polarizing conditions. Functional polarization of Th1, Th2, and Th17 cells, i.e., the cytokine expression profile, was confirmed by intracellular immunofluorescence (Fig. S1). (A) *Twist1* mRNA in resting cells 6 d after the last stimulation (Th1; circles) or after 3 h of restimulation with anti-CD3/CD28 and IL-2 (Th1; squares) or PMA/ionomycin and IL-2 (Th1; triangles: Th2; diamonds) was determined by RT-PCR and normalized to hypoxanthine guanine phosphoribosyl transferase (HPRT). (B) Kinetics of *twist1* mRNA expression in 6-d-old (open bars) and 24-d-old (shaded bars) Th1 cells, after stimulation with anti-CD3/CD28 and IL-2 or IL-2 alone. (C) *Twist1* transcript levels in 6-d-old Th1, Th2, and Th17 cells after reactivation with PMA/ionomycin. (D) Twist1 protein expression in resting (-) and 5 h PMA/ionomycin-restimulated (+) 6- and 24-d-old Th1 and Th2 cells. Control: α-Tubulin immunoblot (bottom). (E) Kinetics of Twist1 protein expression in 24-d-old Th1 cells, before and at the indicated time intervals of stimulation with anti-CD3/CD28 and IL-2. Data are representative of two experiments. Fig. S1 is available at http://www.jem.org/cgi/content/full/jem.20072468/DC1.

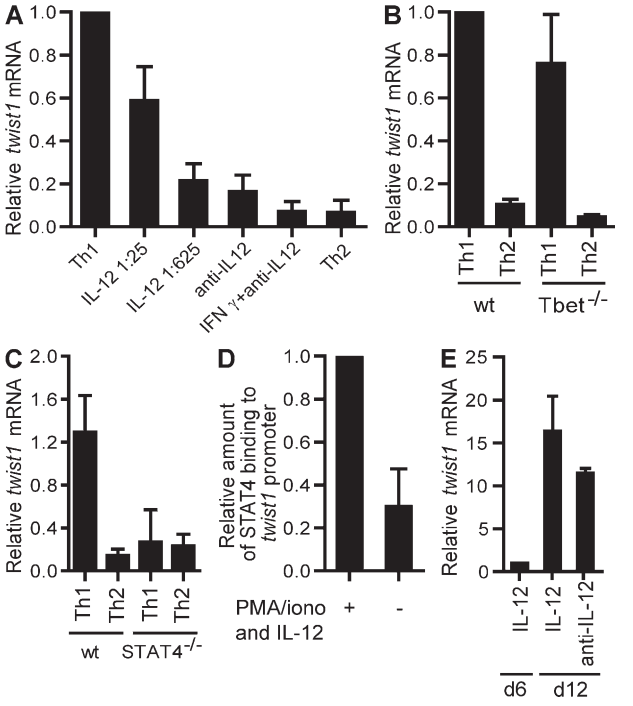


Figure 2. *Twist1* induction requires IL-12 signaling via STAT4, but not IFN-γ or T-bet. (A) $CD62L^{hi}$ DO11.10 Th cells were stimulated for 5 d under Th1-polarizing conditions (5 ng/ml IL-12, anti-IL4), reduced IL-12 (1/25:200 pg/ml; 1/625:8 pg/ml, anti-IL4), in the absence of IL-12 (anti–IL-12, anti-IL4), in the presence of IFN-γ (10 ng/ml IFN-γ, anti-IL-12, anti-IL-4), or under Th2-polarizing conditions. *Twist1* mRNA in Th cells activated for 3 h with PMA/ionomycin and IL-2 was quantified by RT-PCR. The amount of *twist1* transcripts induced under Th1-polarizing conditions was set to 1. Data are presented as the mean ± the SD of at least three experiments. (B) $CD62L^{hi}$ Th cells of T-$bet^{-/-}$ mice and syngenic BALB/c mice were stimulated with anti-CD3/CD28 and BALB/c APCs under Th1 (IL-12 and IFN-γ), or under Th2-polarizing conditions for 6 d. Data represent the mean ± the SD of three experiments. The amount of *twist1* transcripts induced in activated wt Th1 cells was set to 1. (C) $CD4^+$ cells of $STAT4^{-/-}$ and syngenic BALB/c mice were stimulated with anti-CD3/CD28, and BALB/c APCs under Th1 (IL-12 and IFN-γ) or under Th2-polarizing conditions for 5 d. Data represent the mean ± SD (four mice each). (D) The binding of STAT4 to the proximal promoter of *twist1* was analyzed by ChIP. 6-d-old Th1 cells were restimulated with PMA/ionomycin in the presence of 10 ng/ml IL-12 for 3 h or left unstimulated. The immunoprecipitated DNA was quantified by RT-PCR using primers specific for the proximal *twist1* promoter. The precipitated DNA was normalized to the amount of input DNA. The amount of *twist1* transcripts precipitated in the presence of IL-12 was set to 1. Data represent the mean ± SD of three experiments. (E) Naive DO11.10 Th cells were stimulated for 5 d with APCs and $OVA_{327-339}$ under Th1-polarizing conditions. Cells were restimulated under the same conditions (Th1), or in the presence of anti–IL-12. *Twist1* transcripts were quantified on d 11. The amount of *twist1* mRNA on d 5 was set to 1. Data represent mean ± SD of three experiments.

and wild-type Th cells (Fig. 2 B). Also, ectopically expressed T-bet did not induce *twist1* expression in the absence of IL-12 in activated Th cells (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20072468/DC1).

When $CD4^+$ T lymphocytes of STAT4-deficient mice were stimulated in the presence of IL-12 and IFN-γ, no induction of *twist1* expression was detectable (Fig. 2 C). Phylogenetic comparison of the proximal promoter of *twist1* from man and mouse revealed a conserved STAT binding site at position −117 to −137 (Fig. 3 A). STAT4 did bind to the proximal promoter of *twist1* in activated Th1 cells in the presence of IL-12 as shown by chromatin immunoprecipitation (ChIP; Fig. 2 D).

Published July 28, 2008

IL-12/STAT4 is required for the initial induction of *twist1* expression and apparently imprints Th1 cells for reexpression of *twist1* upon reactivation by antigen only. Upon restimulation of Th1 cells, cells cultured in the absence of IL-12 showed a 12-fold increase in the expression of *twist1*, as compared with a 16-fold increase in the presence of IL-12 (Fig. 2 E). IL-4/STAT6 signaling could not suppress *twist1* expression in residual IFN-γ–expressing cells of Th2 cultures (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20072468/DC1), but also did not induce *twist1* expression in activated Th2 cells, as described in Fig. 1.

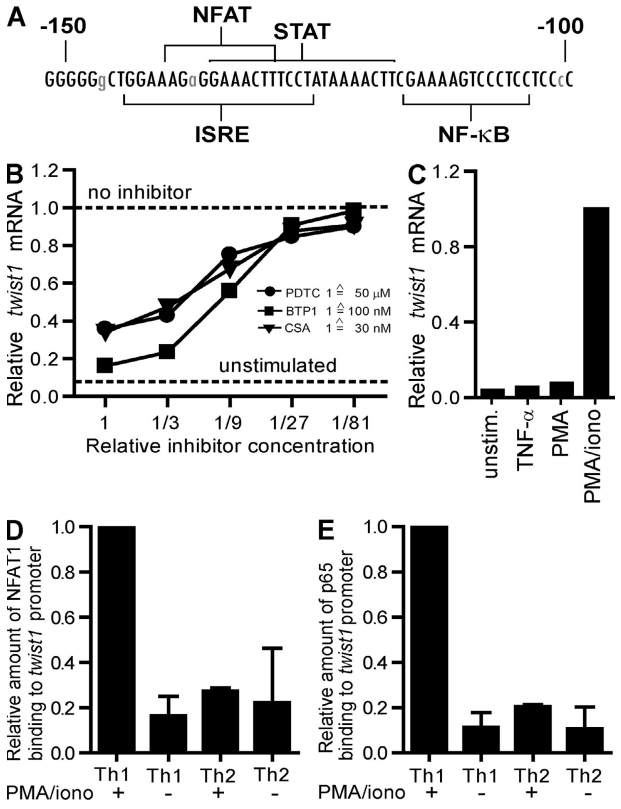


Figure 3. Signaling through NFAT and NF-κB is required for induction of *twist1* expression. (A) Comparison of the genomic sequence of the murine and the human proximal *twist1* promoter (−150 to −100). The murine sequence is displayed with conserved bases in capital letters. Selected putative DNA-binding motifs are indicated. ISRE, IFN-stimulated response element. (B) *Twist1* mRNA in 24-d-old Th1 cells restimulated for 3 h with PMA/ionomycin and IL-2 in the presence of serial dilutions of the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC; circles; starting concentration 50 μM), the NFAT inhibitor BTP1 (squares; starting concentration 100 nM), or the NF-κB and NFAT inhibitor cyclosporine A (CsA; triangles; starting concentration 30 nM). (C) 24-d-old Th1 cells were restimulated in the presence of IL-2 for 3 h with IL-2 alone (unstimulated) or 10 ng/ml TNF-α, PMA, or PMA/ionomycin. The binding of NFAT1 (D) and the NF-κB subunit p65 (E) to the proximal promoter of *twist1* was analyzed by ChIP. 18–24-d-old Th1 and Th2 cells either in the resting state (-) or after restimulation with PMA/ionomycin and IL-2 for 1 h (+) were used. The immunoprecipitated DNA was quantified by RT-PCR using primers specific for the proximal promoter. The precipitated DNA was normalized to the amount of input DNA. The amount of *twist1* transcripts precipitated in activated Th1 cells was set to 1. Data represent mean ± SD of 3 experiments.

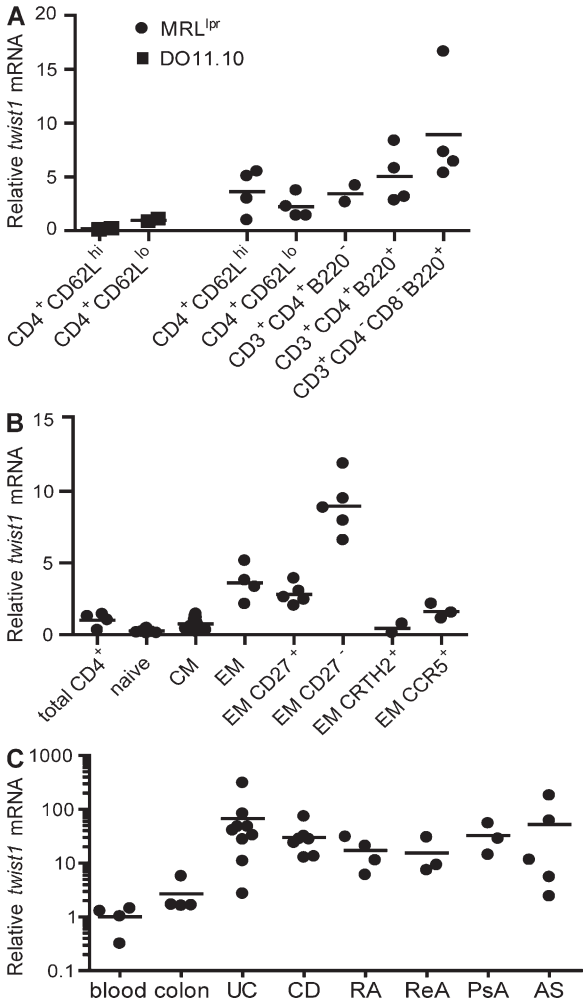


Figure 4. Ex vivo isolated memory Th cells express *twist1*. Cells were sorted by flow cytometry and restimulated for 3 h with PMA/ionomycin (A–C). (A) Cells were isolated from the spleen and lymph nodes of 8–12-wk-old DO11.10 mice (squares, each representing a pool of 15 individual mice) and the pooled inguinal and mesenteric lymph nodes of 4–6-mo-old nephritic MRL/*lpr* mice (circles, 1–2 mice each). Of note: 90% of the $CD4^+CD62L^{hi}$ cells in MRL/*lpr* mice represented activated ($CD44^+$) cells (not depicted). (B) *Twist1* mRNA in peripheral human Th lymphocytes. The mean expression of *twist1* mRNA normalized to ubiquitin ligase H5 in total $CD3^+CD4^+$ cells was set to 1. Subpopulations were defined according to expression of the following surface markers: naive ($CD4^+CD45RA^+CCR7^+$), CM ($CD4^+CD45RA^-CCR7^+$), EM ($CD4^+CD45RA^-CCR7^-$) with each data point representing one individual healthy donor. (C) *Twist1* transcripts in $CD3^+CD4^+$ cells purified from patient material: blood (total peripheral $CD3^+CD4^+$ cells from healthy donors, see B), colon (noninflamed colon tissue), UC, and CD (endoscopic biopsies from UC and CD patients, respectively), RA, ReA, PsA, and AS (synovial fluid from rheumatoid arthritis, reactive arthritis, psoriatic arthritis, and ankylosing spondylitis patients, respectively) with each dot representing one individual patient. Mean *twist1* mRNA expression is displayed from patients who were repeatedly sampled.

Published July 28, 2008

Because *twist1* is expressed transiently upon activation of Th1 cells, we investigated whether signals from the TCR are involved in *twist1* expression control. Phylogenetically conserved binding sites for the TCR signal transducers NF-κB and NFAT are located in the *twist1* promoter at positions −104 to −116 and −129 to −140 (Fig. 3 A). The NF-κB inhibitor pyrrolidine dithiocarbamate (20), the NFAT-specific inhibitory 3,5-bistrifluoromethyl pyrazole derivative BTP1 (21), and the calcineurin-inhibitor cyclosporine A (22) all blocked the PMA/ionomycin-induced expression of *twist1* mRNA (Fig. 3 B), showing that both NFAT and NF-κB are required for induction of *twist1* expression in Th1 cells. Accordingly, stimulation with either TNF-α or PMA alone, both activating NF-κB (20) but not NFAT, did not induce *twist1* expression, whereas PMA in combination with the NFAT-activating ionomycin did (Fig. 3 C).

Using ChIP, the specific binding of NFAT1 and the transactivating NF-κB subunit p65 to the *twist1* promoter of repeatedly stimulated Th1 cells was evident 1 h after reactivation (Fig. 3, D–E). In Th2 cells, no activation-induced binding of NFAT1 or NF-κB to the *twist1* promoter was detectable, emphasizing that NFAT1 and NF-κB are required, but not sufficient, to induce transcription of *twist1* in Th cells. For the initial expression of *twist1* in Th1 cells, and its imprinting for reexpression in further restimulations, the concerted action of activated STAT4, NFAT1, and NF-κB is required. Imprinting of the *twist1* gene for reexpression in Th1 cells is reflected by increased acetylation of histone H3 and trimethylated histone H3 at lysine 4, in both resting and reactivated Th1 cells, as compared with Th2 cells (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20072468/DC1).

Th cell–specific *twist1* expression in vivo

The exclusive expression of *twist1* in Th1 EM cells generated in vitro is reflected by the expression pattern of *twist1* in Th cells isolated ex vivo. Activated $CD4^+CD62L^{hi}$ naive and $CD4^+CD62L^{lo}$ memory cells isolated from the spleen and lymph nodes of healthy DO11.10 mice, kept under specific pathogen–free conditions, expressed low levels of *twist1* (Fig. 4 A). In $CD4^+CD62L^{lo}$ memory Th cells from lymph nodes of 4–6-mo-old nephritic lupus-prone MRL/*lpr* mice (23) *twist1* mRNA was up-regulated by approximately twofold, as compared with $CD4^+CD62L^{lo}$ Th cells from DO11.10 spleen (Fig. 4 A). *Twist1* expression was enhanced by five- to eightfold in $CD3^+B220^+$ T cells from MRL/*lpr* mice, which represent chronically activated T lymphocytes (24, 25).

In analogy to murine Th lymphocytes, human Th cells can express *twist1*. *Twist1* expression was enhanced by threefold in EM (i.e., $CD45RA^-CCR7^-$) Th cells, and eightfold in "terminally" differentiated $CD27^-$ EM Th cells (26, 27), whereas naive ($CD45RA^+CCR7^+$) and central memory (CM; i.e., $CD45RA^-CCR7^+$) Th lymphocytes expressed less *twist1* than unseparated Th cells. Activated Th1 EM cells, characterized by the expression of CCR5 (28), expressed *twist1*, whereas Th2 EM cells characterized by expression of chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTh2) (29) did not (Fig. 4 B).

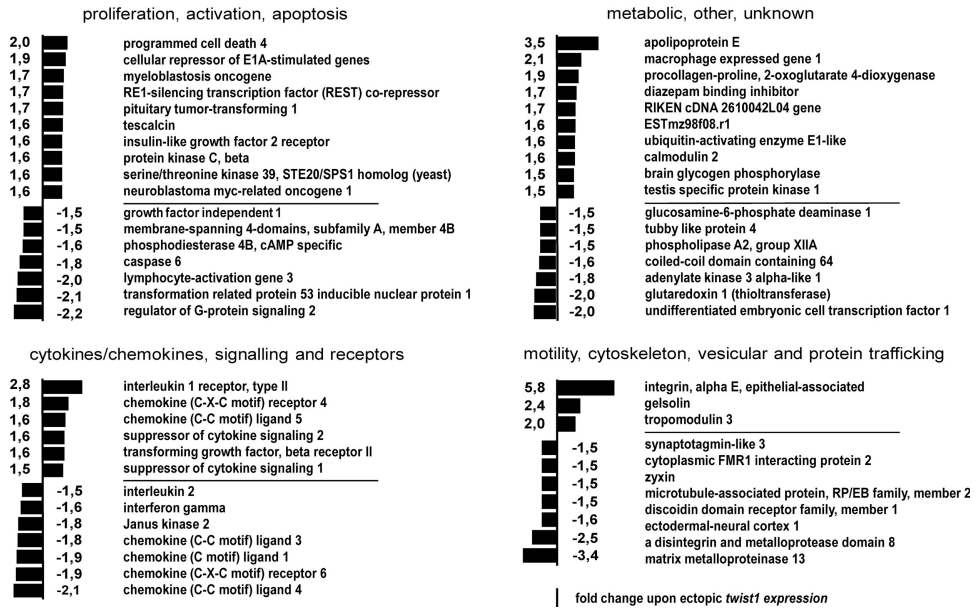


Figure 5. Genes differentially expressed upon ectopic *twist1* overexpression. Splenic DO11.10 cells were activated in vitro with the cognate peptide $OVA_{327-339}$ in the presence of 1 ng/ml IL-12 and 1 ng/ml IL-2. On d 2, cells were infected with control retrovirus, or *twist1*-encoding virus. On d 5, cells were sorted according to expression of the viral marker gene *gfp*. Cells were restimulated for 4 h with PMA/ionomycin. The transcriptional profiles of duplicates of cultures were compared.

Published July 28, 2008

Th cells isolated from inflamed tissue of patients with chronic inflammatory diseases are imprinted for high *twist1* expression. Although highly variable, *twist1* transcripts were increased by up to 400-fold, as compared with peripheral Th cells, in $CD3^+CD4^+$ cells isolated from the synovial fluid of inflamed joints of patients with rheumatoid arthritis or spondyloarthropathies, and in Th cells isolated from mucosal endoscopic biopsies and surgical specimens of patients suffering from CD or UC (Fig. 4 C and Table S2, available at http://www.jem.org/cgi/content/full/jem.20072468/DC1). *Twist1* mRNA expression in repeatedly sampled patients with persistent inflammation of colon or synovia remained in the same range over up to 18 mo (Table S2). Among T cells isolated from the inflamed tissue, only $CD4^+$ Th cells showed enhanced *twist1* expression. $CD3^+CD4^-$ cells, i.e., cytotoxic (Tc) lymphocytes, expressed *twist1* transcript levels lower than the reference value of total peripheral Th cells (unpublished data).

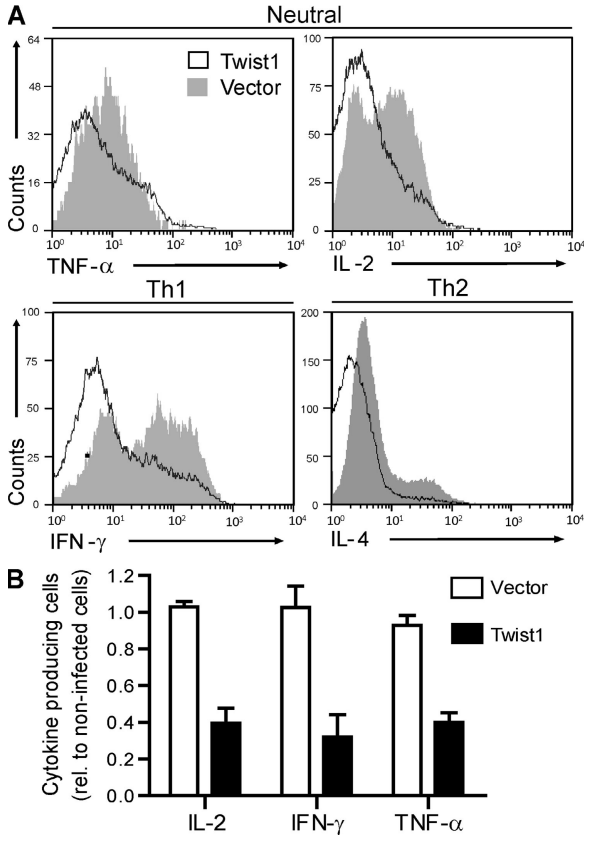


Figure 6. *Twist1* suppresses the expression of effector cytokines. DO11.10 Th cells were stimulated for 5 d under Th1- or Th2-polarizing conditions or without addition of cytokines. On d 2, cells were infected with control virus or *twist1*-encoding virus. The cells were restimulated on d 6 and stained for intracellular cytokine expression. (A) Representative histograms of cytokine expression in Th cells ectopically expressing *twist1* (black line) and control cells (gray filled). The cells displayed were gated for expression of CD4 and the viral marker gene *gfp*. (B) Frequencies of cytokine-expressing cells among infected, i.e., GFP^+CD4^+ T cells relative to the noninfected $CD4^+$ cells. Cells had been stimulated without addition of cytokines and infected with control virus (open bars) or *twist1*-encoding virus (filled bars). Data represent the mean ± SD of four independent experiments.

Functional modulation of Th1 cells by *twist1*

We analyzed the impact of *twist1* on the function of Th1 EM cells by ectopic overexpression of *twist1* in murine DO11.10 Th cells. A global view of *twist1*-induced modulation of gene expression in Th1 cells is provided in Fig. 5. Of the 14,000 genes analyzed for transcription, 58 were differentially expressed by a factor of 1.5 or more when comparing activated Th1 cells that express *twist1* ectopically and those that do not (Fig. 5). These genes fall into 4 groups, with respect to their presumptive function: 17 genes are involved in cell activation and apoptosis, 11 genes are involved in cell adhesion and motility, 13 genes relate to the chemokine/cytokine repertoire of Th1 cells, and 17 genes are of metabolic or undefined relevance.

Of relevance for the effector function of Th1 EM cells, *twist1* attenuated expression of the effector cytokine genes *il-2, ifn-γ*, and *tnf-α* by a factor of up to 1.6 (Fig. 5 and Table S3, available at http://www.jem.org/cgi/content/full/jem.20072468/DC1). The moderate reduction of mRNA levels of effector cytokines had a drastic effect on protein expression regardless of the direction of Th cell differentiation. Ectopic *twist1* expression reduced the frequencies of cytokine-expressing reactivated Th1 or Th2, or of Th cells that had been stimulated without addition of polarizing cytokines to the culture, to 40–50% of the controls (Fig. 6 and Fig. S5).

The molecular basis of the regulation of cytokine gene expression by *twist1* could be either direct inhibition of NF-κB, as has been shown for COS cells (4), or binding of Twist1 to E-boxes of regulatory elements of the cytokine genes, blocking activating transcription factors, as has been shown for primary macrophages (5). Here, we show that ectopic *twist1* expression in activated Th cells cannot inhibit activation-induced transcription of an NF-κB–reporter construct lacking E-boxes, i.e., Twist1 does not inhibit NF-κB directly (Fig. 7 A), in contrast to the constitutively active mutant form of *inhibitor of NF-κB* (*I-κBαM*), in an experimental situation where both Twist1 and I-κBαM are expressed at similar levels (Fig. S6, available at http://www.jem.org/cgi/content/full/jem.20072468/DC1), and both attenuate the expression of endogenous cytokine genes to the same degree (Fig. 7 B). This result implies that *twist1* regulates gene expression of Th1 cells by binding to E-boxes of specific target genes, and not the entirety of NF-κB–regulated genes. NF-κB signaling for the generation and survival of Th1 memory cells (30, 31) is not inhibited by *twist1*, as is also evident from the high *twist1* expression of repeatedly restimulated Th1 cells (Fig. 1).

Twist1 regulates Th1-mediated inflammation

In a murine transfer model of OVA-specific DTH, the effect of *twist1* expression on the inflammation induced by transferred Th1 cells was analyzed. OVA-specific 6-d-old Th1 cells ectopically overexpressing *twist1* and control Th1 cells were transferred intravenously into naive BALB/c mice.

Published July 28, 2008

After 1 d, $OVA_{323-339}$ emulsified in IFA was injected into one footpad and the swelling of this footpad was monitored. Th1 cells overexpressing *twist1* showed a significantly ($P < 0.05$) reduced induction of footpad swelling, starting from day 4 to 7, when compared with control Th1 cells (Fig. 8 A). For DTH mediated by Th1 cells the proinflammatory role of Th1-derived cytokines such as IFN-γ has been shown (32). Transferred Th cells overexpressing *twist1*, when reisolated from the draining lymph nodes of the host, expressed fourfold reduced levels of IFN-γ mRNA as compared with the control cells (Fig. 8 B). Homing of transferred Th1 cell populations to the inflamed tissue and the draining lymph nodes was not affected by *twist1* overexpression, as shown by the transfer of radiolabeled cells (Fig. 8 C). The accumulation of ectopically *twist1*-expressing Th1 cells in the draining lymph nodes and the inflamed foot pads, as well as in other organs (Fig. S7, available at http://www.jem.org/cgi/content/full/jem.20072468/DC1), was comparable to that of transferred control Th1 cells.

Autoregulation of Th1-mediated inflammation by *twist1* was also analyzed in a murine adoptive transfer model of antigen-induced arthritis (Fig. 9 A). In this model, we used the complementary genetic approach to knockdown *twist1* expression in Th1 cells by RNA interference. Murine DO11.10 Th1 cells generated in vitro were infected with a retrovirus encoding a small hairpin RNA (shRNA) targeting *twist1* (3) or a corresponding scrambled control shRNA. *Twist1*-specific shRNA reduced the level of activation-induced endogenous *twist1* transcripts in Th1 cells to ∼30% of the control value (Fig. 9 B). Two-time restimulated Th1 cells expressing those shRNAs were intravenously injected into SCID mice. 1 d after cell transfer, arthritis was induced by injection of cationized OVA into the knee joint, and histological analysis was performed 3 wk after induction of arthritis, i.e., in the chronic phase of inflammation. *Twist1* knockdown and control Th1 cells equally homed to and persisted in the spleen and mesenteric and draining lymph nodes (Fig. 9, C–E). However, *twist1* knockdown in Th1 cells resulted in a significantly higher histological score of inflammation and tissue destruction (Fig. 9 F and Table S4, available at http://www.jem.org/cgi/content/full/jem.20072468/DC1). In particular, infiltration of granulocytes and mononuclear cells into the inflamed tissue of the knee joint was drastically enhanced (Fig. 10 and Fig. S8), as were characteristics of chronic inflammation, i.e., prominent hyperplasia of the lining cells, pannus formation, increased vascularization, and hyperplasia of synovial fibroblasts in the sublining layer (Fig. 10 and Fig. S8). Increased numbers of $Gr\text{-}1^+$ granulocytes at the surface of the synovium and focal accumulation of $F4/80^+$ macrophages were observed after transfer of *twist1* knockdown Th1 cells as compared with control Th1 cells (Fig. S8). Anti-TNF-α staining of joint sections revealed stronger TNF-α expression, especially in the lining cells and the sublining layer after transfer of Th1 cells expressing a *twist1*-targeting shRNA (Fig. 10). Presumably as an additional consequence of the higher inflammation in the mice receiving *twist1* knockdown Th1 cells, more $CD3^+$ T cells were found in the inflamed joints (Fig. 10).

DISCUSSION

Control of inflammation critically depends on the *twist* genes. Genetic haploinsufficiency of both *twist1* and *twist2* results in fatal systemic inflammatory immunopathology in mice, which die before day 14 (4). In this study, we show that *twist1* is expressed in Th1 EM cells. Its expression is induced by IL-12

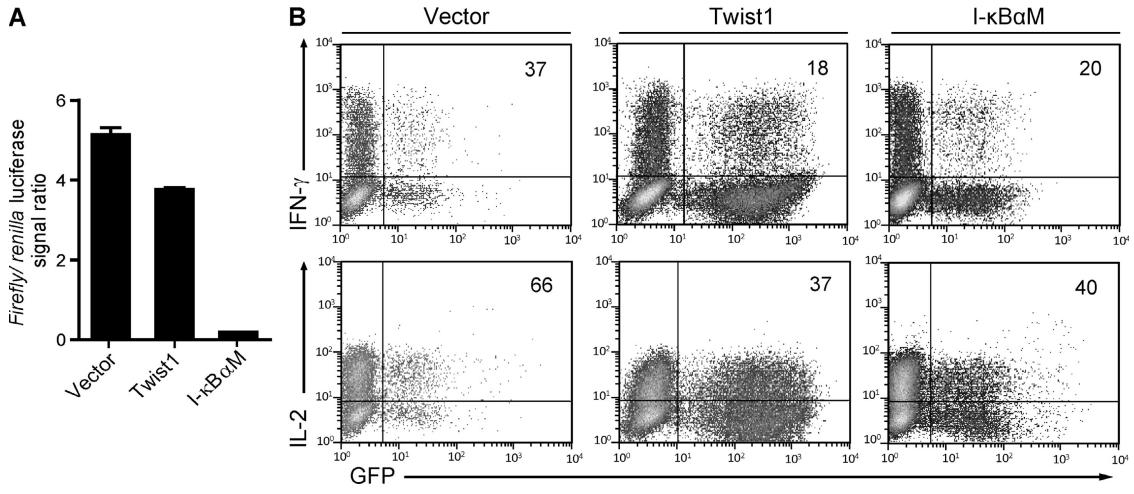


Figure 7. Inhibition of NF-κB–mediated signaling by *twist1* is promoter-specific. DO11.10 Th cells were stimulated with $OVA_{327-339}$, APCs, and 1 ng/ml IL-12. On d 2, cells were infected with control virus, *twist1*, or I-κBαM-encoding virus. On d 3, cells were nucleoporated with a mixture of a plasmid encoding *Renilla luciferase*, and a *firefly luciferase* reporter construct, driven by a NF-κB–responsive promoter (4xκB-luc). (A) Cells were restimulated with PMA/ionomycin for 6 h, sorted according to expression of the viral marker gene *gfp*, and luciferase signals were quantified in duplicates (mean ± SD). (B) The very same Th1 cultures were restimulated on d 5 and stained for intracellular cytokine expression. Frequencies of cytokine-expressing cells among infected, i.e., GFP^+CD4^+ T cells, are displayed. Data are representative of two experiments.

Published July 28, 2008

via STAT4 and TCR signaling, activating NFAT and NF-κB. Expression of *twist1* follows TCR stimulation transiently and increases upon repeated stimulation. Thus, imprinting for enhanced *twist1* expression is a hallmark of repeatedly restimulated Th1 memory cells.

The proximal promoter of *twist1* contains phylogenetically conserved binding sites for NFAT, NF-κB, and STAT proteins. Both NFAT and NF-κB have to bind to the promoter of *twist1* in Th cells to induce expression, i.e., *twist1* is expressed only by activated Th cells. In the initial activation of naive Th cells, NFAT and NF-κB cannot induce transcription of *twist1* on their own, but require concerted binding of activated STAT4 to the promoter of *twist1*. Of all costimulatory signals involved in the lineage differentiation of murine Th1, Th2, or Th17 cells, only IL-12 was able to induce transcription of *twist1*. Neither IL-4, in Th2 polarization, nor IL-6, TGF-β, and IL-23 in Th17 polarization, could induce expression of *twist1*. Of the Th1-polarizing signals, IL-12/STAT4, but not IFN-γ/STAT1, is required to induce *twist1* expression. T-bet is not involved. STAT4 is required only in the original stimulation, to imprint the gene for reexpression. This imprinting is evident from the increased reexpression upon stimulation with TCR signals alone, in the absence of IL-12, and it is reflected in the acetylation and trimethylation of H3 histones at the *twist1* promoter region, as is shown here. The requirement of NFAT for induction of *twist1* expression in Th1 cells distinguishes control of *twist1* expression in Th cells from its control in fibroblasts, where TNF-α–induced activation of NF-κB is sufficient to induce expression (4). In activated macrophages, type I IFNs induce the expression of *twist1* (5), and they express both *twist* genes, i.e., *twist1* and *twist2*, whereas activated Th1 cells exclusively express *twist1*. In Th lymphocytes, *twist1* expression is not induced by type I IFNs. Addition of IFN-α during the primary activation of Th cells in the absence of IL-12 did not suffice to induce *twist1* expression (Fig. S9, available at http://www.jem.org/cgi/content/full/jem.20072468/DC1).

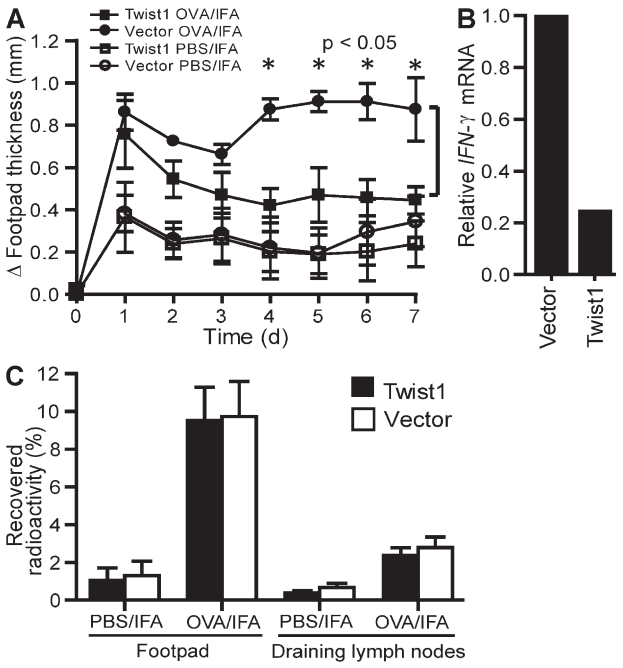


Figure 8. Ectopic *twist1* overexpression controls DTH. (A) Naive DO11.10 Th cells were stimulated under Th1-polarizing conditions. On d 2, cells were infected with control virus (circles) or *twist1*-encoding virus (squares). On d 6, infected GFP+ cells were injected i.v. into BALB/c mice. The DTH response was induced by s.c. $OVA_{323-339}$/IFA injection into the left footpad (filled symbols), and Δ footpad thickness (mean ± SD; $n = 4$, Mann-Whitney test, nonparametric) was determined thereafter. Injection of PBS/IFA served as control (open symbols). (B) Ex vivo IFN-γ mRNA expression in transferred GFP+ Th1 cells 24 h after DTH induction isolated from the draining popliteal lymph node (left foot). (C) To monitor the migratory capacity of the transferred cell populations, infected GFP+ Th cells were radiolabeled and injected i.v. into BALB/c mice 1 d after induction of the DTH response. 24 h later radioactivity recovered from indicated tissues was determined using a γ-counter (mean ± SD; $n = 4$).

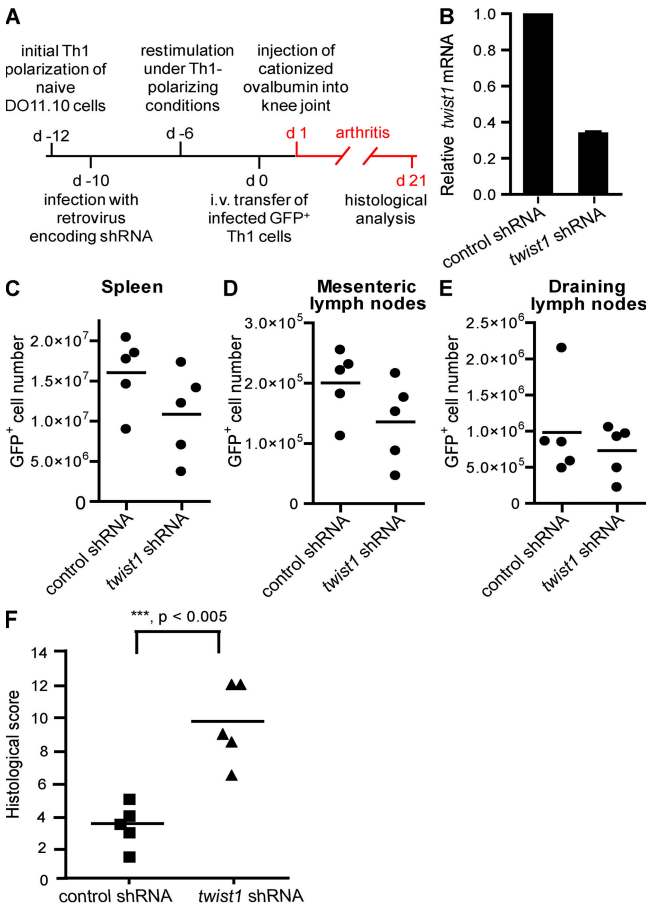


Figure 9. *Twist1* knockdown increases inflammatory response in murine arthritis. (A) Experimental scheme. (B) *Twist1* mRNA in 18-d-old Th1 expressing *twist1*-targeting shRNA or control shRNA restimulated with PMA/ionomycin. (C–E) Cell numbers of adoptively transferred GFP+ Th cells in spleen, mesenteric lymph nodes, and draining lymph nodes were analyzed on d 21 using FlowCount Beads. (F) Transfer of Th1 cells expressing a *twist1*-targeting shRNA leads to a significantly higher histological score in murine arthritis compared with control Th1 cells (d 21). Data are representative of two experiments.

Published July 28, 2008

These findings provide evidence that expression of the anti-inflammatory *twist* genes is controlled by type I IFNs and TNF-α in fibroblasts and macrophages in innate immune responses (4, 5), and is controlled by antigen and IL-12 in Th cells in adaptive immune responses; i.e., *twist* expression is induced by those cytokines controlling the induction of inflammatory immune responses.

In this study, we have identified *twist1* as a gene that is differentially expressed by Th1 cells versus Th2 cells, comparing the transcriptomes of once and repeatedly restimulated murine Th1 and Th2 cells generated in vitro from bona fide naive Th cells. Expression of *twist1* increases upon repeated restimulation in vitro in Th1 cells, whereas it is not induced even in repeatedly restimulated Th2 or Th17 cells. Accordingly, in Th cells isolated ex vivo, *twist1* expression is restricted to a subset of memory cells. Among murine $CD4^+CD62L^{lo}$ memory-phenotype Th cells that were isolated from spleen or lymph nodes of naive DO11.10 mice, expression of *twist1* is low. In $CD3^+B220^+$ T cells isolated from nephritic MRL/*lpr* mice, which represent chronically activated T cells (24, 25), *twist1* expression is up-regulated five- to eightfold compared with DO11.10 memory-phenotype Th cells. This is moderate when compared with the ∼20-fold up-regulation upon repeated restimulation of Th1 cells observed in vitro, and may reflect the heterogeneous composition of the cell populations analyzed. As expected from the phylogenetic conservation of the *twist1* promoter, *twist1* is also expressed by activated human Th cells. It is low in peripheral human naive Th cells and in $CCR7^+$ CM Th cells. *Twist1* expression is enhanced in $CCR7^-$ EM Th cells, in particular in the "terminally differentiated" $CCR7^-CD27^-$ EM cells (26, 27). Among $CCR7^-$ EM cells, *twist1* is expressed less by $CRTh2^+$ Th cells, which have been shown to be mostly Th2 cells (29). $CCR7^-CCR5^+$ EM cells, which contain Th1 EM and Th17 EM cells (33) have up-regulated activation-induced expression of *twist1*, as we show here. Because at least Th17 cells generated in vitro do not express *twist1*, the expression in $CCR7^-CCR5^+$ Th EM cells is probably caused by Th1 EM cells. Thus, the phenotype of human peripheral *twist1*-expressing Th cells is that of repeatedly restimulated Th1 EM cells. Remarkably, expression of *twist1* is highly up-regulated in $CD3^+CD4^+$ T cells isolated from inflamed tissues of patients with chronic inflammation of joints or gut. Repeated biopsies from individual patients show persistent *twist1* mRNA levels over time, i.e., the persistence of chronically reactivated Th1 cells, despite state-of-the-art therapeutic treatment. Their imprinting for increased *twist1* expression, maintained over time in individual patients, indicates a history of repeated restimulation and an endogenous regulation of proinflammatory effector functions under the control of NFAT and NF-κB.

Twist1 itself is a transcriptional repressor binding to E-boxes in the regulatory regions of target genes (34). For COS cells, it has been shown that ectopically expressed *twist1* can interact directly with the p65 subunit of NF-κB and inhibit its function (4). In contrast, for primary murine macrophages, *twist1* has been shown to block transcription of target genes by binding to E-boxes within the promoter (5). In this study, we show that in Th1 cells, *twist1* cannot block NF-κB–driven transcription of a reporter gene construct that lacks E-boxes in the promoter, suggesting that *twist1* acts through binding to E-boxes, as in macrophages. In accordance, ectopically expressed *twist1* only regulates the expression of a restricted set of 58 genes in 1-wk-old Th1 cells by a factor of at least 1.5. Expression of 29 genes is up-regulated, and that of another 29 genes down-regulated. Apart from several genes of unknown and metabolic function, these genes are involved in survival, effector function, and motility of the cells. With respect to cytokine expression, *twist1* reduces activation-induced expression of TNF-α, IL-2, and IFN-γ by Th1 memory cells by >50% at the protein level. Apart from cytokine expression, *twist1* also decreases expression of proinflammatory chemokines

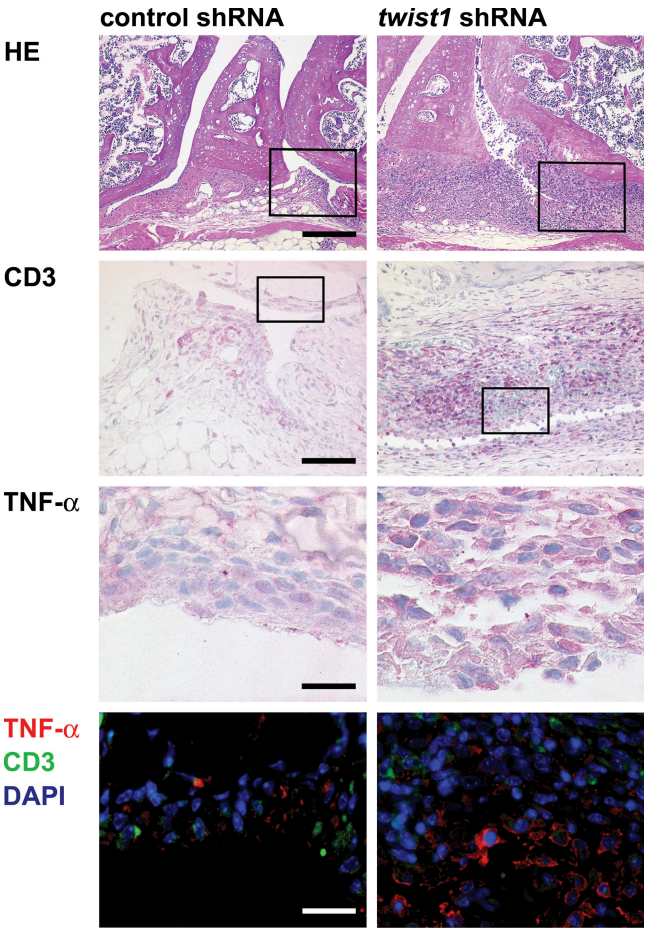


Figure 10. *Twist1* knockdown leads to pronounced signs of chronic inflammation in murine arthritis. Representative hematoxylin/eosin staining of knee joint sections (d 21) showing hyperplasia of the lining cells and the subintimal layer with pannus formation (bar, 200 µm), more pronounced infiltration of $CD3^+$ T cells (magnification of boxed areas in top row; bar, 100 µm) and stronger expression of TNF-α (red) especially of the enlarged lining and sublining layer (magnification of boxed areas in second row; bar, 50 µm) after transfer of Th1 cells expressing a *twist1*-targeting shRNA as compared with the control group. Data are representative of five mice each.

Published July 28, 2008

and chemokine receptors, and attenuates cytokine receptor signaling by up-regulating SOCS-1 and -2, the IL-1 decoy receptor, and by down-regulating Jak2, the kinase involved in IL-12 signaling. *Twist1* in Th1 cells thus acts as an endogenous regulator limiting the proinflammatory potential of Th1 cells in the continuous presence of antigen, i.e., repeated activation of NFAT and NF-κB.

The key role of *twist1* expression by Th1 cells for the self-limitation of Th1-induced inflammation is evident from the present analysis of murine models of inflammation. Ectopic overexpression of *twist1* in 6-d-old Th1 cells, which still had a low endogenous expression level, drastically reduced their pathogenic contribution to DTH. Conversely, shRNA-mediated knockdown of *twist1* in Th1 cells significantly enhanced their potential to induce chronic inflammation in a murine model of antigen-induced arthritis. In this model, OVA-specific Th1 cells, when adoptively transferred into SCID mice and challenged by intraarticular OVA, induce a chronic inflammation of the joint. Adoptive transfer of Th1 cells versus Th1 cells with a shRNA-mediated knockdown of *twist1* expression demonstrates the relevance of *twist1* expression by Th1 cells for the control of immunopathology. When analyzed on day 21 after transfer, mice with a knockdown of *twist1* expression in Th1 cells showed increased hyperplasia of the lining cells and synovial fibroblasts in the sublining layer, pannus formation, and vascularization. Numbers of Gr-1$^+$ granulocytes and F4/80$^+$ macrophages, as well as TNF-α expression of lining and sublining cells, were increased in the Th1 *twist1* knockdown situation.

Why is expression of *twist1* up-regulated gradually? One explanation might be that a regulatory gene is not expressed in initial Th1 activations, to allow full initial reactivity of the Th1 cells. Accordingly, ectopic *twist1* expression in Th1 cells right from the beginning impairs their function in acute inflammation of DTH. In repeated restimulations, reflecting a lack of clearance of the antigen and increased risk of immunopathology, regulation of Th1 may help to limit immunopathology, as we show in the chronic inflammation of the antigen-induced arthritis model by knockdown of *twist1*, but *twist1*-expressing Th1 cells are still capable of driving inflammation.

Apart from its role in limiting immunopathology in chronic inflammation, *twist1* and the genes controlled by it could be regarded as biomarkers for pathogenic EM cells. *Twist1* is not imprinted for enhanced expression in naive and CM CCR7$^+$ Th cells. Even CCR7$^-$CCR5$^+$ Th1 EM cells will only express a low level of *twist1* upon restimulation compared with Th cells from chronically inflamed tissue. Initial clinical studies aiming at a complete depletion of CD4$^+$ T lymphocytes in patients with chronic inflammatory diseases suggested a clinical benefit, but the studies had to be terminated because of the severe side effects of systemic depletion of Th lymphocytes (35–37). Targeting of CD4- and CD3-expressing cells with nondepleting antibodies and neutralization of Th-related effector cytokines have demonstrated clinical efficacy (12, 13, 15, 38–40), but all of them impair protective as well as pathogenic T cell memory. Targeting of *twist1*-expressing Th cells, by means of either addressing *twist1* or a gene regulated by it, seems more promising, because it is more specific for repeatedly restimulated Th1 memory cells involved in chronic inflammation. It remains to be shown whether these cells in chronic inflammatory diseases are the critical Th cells capable of driving inflammation, as we show in this study for the experimental model of antigen-induced arthritis.

MATERIALS AND METHODS

Mice and reagents. MRL$^{lpr/lpr}$, BALB/c, C57BL/6, SCID, STAT4-deficient mice (Stat4^{tm1Gr}), and OVA-TCR$^{tg/tg}$ DO11.10 mice were purchased from The Jackson Laboratory or were bred under specific pathogen–free conditions in our animal facility. T-bet–deficient mice were a gift from J. Penninger (Institute for Molecular Biotechnology of the Austria Academy of Sciences, Vienna, Austria). All animal experiments were performed in accordance with institutional, state, and federal guidelines (Landesamt Für Gesundheit und Soziales, Berlin, Germany). Reagents were purchased from Sigma-Aldrich unless otherwise stated. BTP1 was synthesized by M. Paetzel (Humboldt University of Berlin, Berlin, Germany). Cyclosporin A was purchased from Calbiochem.

Patients. Endoscopic mucosal and surgical mucosal specimens were obtained from UC (n = 9) and CD (n = 7) patients. UC and CD were diagnosed according to established clinical, endoscopic, radiological, and pathological criteria. All UC and CD patients displayed moderately to severely active disease according to the Truelove and Witts Severity Index (41) and the Harvey Bradshaw Severity Index (42), respectively. Control samples were obtained from patients (n = 4) undergoing colonectomy because of colon cancer. Mucosal control specimens used in the study were from the macroscopically noninvolved tissue distant from any detectable lesion. Synovial fluid was obtained from patients suffering from rheumatic diseases who had active synovitis with effusion. Patients with rheumatoid arthritis (RA; n = 4) fulfilled the American College of Rheumatology 1987 classification criteria for RA, patients with ankylosing spondylitis (AS; n = 5) fulfilled the modified New York criteria (1984), and patients with psoriatic arthritis (n = 3) or reactive arthritis (n = 3) fulfilled the European Spondyloarthropathy Study Group (ESSG) criteria for SpA. Clinical characteristics of the patients are listed in Table S2. All experiments were approved by the local ethics committee (Charite Ethikkommision), and all patients gave informed consent.

Isolation of human lymphocytes. PBMCs from buffy coats from healthy donors, and synovial fluid mononuclear cells were isolated by density gradient centrifugation (Lymphocyte separation medium; PAA). Pooled intraepithelial leukocytes and lamina propria leukocytes were obtained from mucosal specimens by treatment with collagenase type IV, followed by passage through a sieve. Mononuclear cells were collected from the 40–70% interphase of a discontinuous percoll (Pharmacia) gradient.

Flow cytometry. The following antibodies directed against murine antigens were either purified from hybridoma supernatants and conjugated in-house or purchased as indicated: anti-CD3 (145-2C11), anti-CD4 (GK1.5), anti-CD8 (53–6.7), anti-CD11c (N418), anti-CD44 (IM7), anti-CD62L (MEL14), anti-DO11.10 OVA-TCR (KJ1.26), anti-B220 (RA3.6B2), anti-IL2 (JES6-5H4; Caltag Laboratories), anti-IL-4 (11B11; BD Biosciences), anti-IFN-γ (AN18.17.24), and anti-TNF-α (MP6-XT22; Caltag). Antibodies recognizing human antigens were obtained from BD Biosciences unless stated otherwise: anti-CD3 (OKT3; in-house conjugate), anti-CD4 (TT1; in-house conjugate), anti-CD27 (L128), anti-CD45RA (HI100), anti-CRTh2 (BM16), and anti-CCR5 (2D7/CCR5). Cells were counted by using FlowCount Beads (Beckman Coulter). Cytometric analysis was performed with FACSCalibur using CellQuest (BD Biosciences) and FCS Express (De Novo) software. Cells were separated by fluorescence-activated cell sorting (FACSAria and FACSDiva; BD Biosciences).

Published July 28, 2008

Cell culture. Naive $CD4^+CD62L^+$ lymphocytes from 6–8-wk-old DO11.10 mice were isolated and polarized under Th1 or Th2 conditions, as previously described (15). Irradiated (30 Gy) BALB/c splenocytes were used as APCs at a ratio of 5:1 and the cognate peptide $OVA_{323-339}$ (provided by R. Volkmer-Engert, Humboldt University of Berlin, Berlin, Germany) was added at 0.5 μM. Alternatively, plates were coated with 3 μg/ml anti-CD3 (145-2C11) in PBS, and $CD4^+$ cells were plated at a density of 2×10^6 cells/ml in medium plus 1 μg/ml soluble anti-CD28 (37.51). For Th17 differentiation, cells were stimulated in the presence of 1 ng/ml TGF-β (R&D Systems), 20 ng/ml IL-6 (R&D Systems), and 20 ng/ml IL-23 (R&D Systems), as well as 10 μg/ml anti-IL-4 and 10 μg/ml anti–IFN-γ. Irradiated IL-12 $p35^{-/-}$ splenocytes were used as APCs. Every 6 d, viable Th cells were harvested and restimulated under the original conditions, except that 10 ng/ml murine IL-2 (R&D Systems) was added to the Th1 and Th2 cultures.

Mitogenic restimulation and intracellular cytokine staining. Cells were restimulated with 10 ng/ml PMA, and 1 μg/ml ionomycin or with plate-bound anti-CD3 (10 μg/ml), and soluble anti-CD28 (1 μg/ml). For restimulation of murine T cells, 10 ng/ml IL-2 was added. For intracellular staining of cytokines, T cells were stimulated for 2 h with PMA/ionomycin and an additional 3 h with 5 μg/ml of brefeldin A. Cells were fixed with 2% formaldehyde in PBS for 15 min at room temperature and permeabilized with 0.5% wt/vol saponin.

ChIP. ChIP was performed as previously described (15) using anti-NFAT1 (AB1-209; ImmunoGlobe Antikoerpertechnik), anti-p65 (C-20; Santa Cruz Biotechnology, Inc.), anti-STAT4 (C-20; Santa Cruz Biotechnology, Inc.), anti–acetyl-histone 3 (#06–599; Millipore) or anti–trimethyl-K4-histone 3 (#07–473; Millipore), followed by incubation with Protein A microbeads. The relative amount of precipitated DNA was calculated with $E^{\Delta Cp\ (input\ -\ immunoprecipitate)}$. The following primers were used to amplify the proximal *twist1* promoter: (−150 forward) 5′-GGGCTGGAAAGAGGAAACTT-3′; (+4 reverse) 5′-CGCGAGGTGTCTGAGAGTT-3′.

Th1-mediated DTH model. The OVA-specific DTH model was performed as described elsewhere (43). In brief, 5×10^5 Th1 cells were injected i.v. into BALB/c mice, and 24 h later, the DTH response was induced by s.c. injection of 250 ng $OVA_{323-339}$/IFA into the left footpad. PBS/IFA, injected into the right footpad, served as a control. The footpad thickness measured before the injection of the antigen was subtracted from the footpad thickness measured during the DTH response. The homing of adoptively transferred Th1 cells was performed as previously described (44). In brief, Th cells were labeled with ^{51}Chromium (Amersham Buchler) at 37°C (2×10^7 cells/ml; 20 μCi/ml) in fresh medium. Removal of dead cells was done by gradient centrifugation (17.1% isotonic Nycodenz). Labeled cells were coadoptively transferred into recipient animals at 1 d after the DTH induction. 24 h later, indicated tissues were removed and differential measurement of recovered radioactivity was done on a γ counter (Wallac Counter).

Antigen-induced arthritis. 2×10^6 12-d-old GFP^+ Th1 cells were transferred i.v. into naive SCID mice. 1 d later, arthritis was induced by intraarticular injection of 60 μg cationized OVA into one knee joint. The contralateral knee joint was left untreated. 21 d later, mice were killed and knee joints were fixed in 10% formaldehyde, decalcified in saturated EDTA solution, and embedded in paraffin. Knee joint sections were stained with hematoxylin/eosin and scored for exudates, granulocyte infiltration, hyperplasia, fibroblast proliferation/mononuclear cell infiltration, periarticular mononuclear cell infiltration (each scoring 0–3), bone/cartilage destruction (scoring 0–4), and an additional score of 1 for visible fibrin deposition and periarticular granulocyte infiltration, resulting in a maximum score of 21.

Immunohistochemistry. Formalin-fixed paraffin-embedded tissue was subjected to a heat-induced epitope retrieval step and stained with anti-CD3 (#N1580; Dako), TNF-α (PeproTech), anti-Gr-1 (RB6-8C5; eBioscience), or F4/80 (eBioscience). For detection biotinylated donkey anti–rat or donkey anti–goat (Dianova) secondary antibodies were used followed by the streptavidin AP kit (K5005; Dako). Or sections were incubated with goat anti-TNF-α antibody followed by Alexa Fluor 555-conjugated anti–goat antibody (Invitrogen) and incubated with rabbit polyclonal anti-CD3 followed by Alexa Fluor 488–conjugated anti–rabbit antibody (Invitrogen). Nuclei were counterstained with DAPI (Roche), and slides were mounted in Fluoromount-G (SouthernBiotech). Images were acquired using a fluorescence microscope (AxioImager Z1) equipped with a charge-coupled device camera (AxioCam MRm) and processed with Axiovision software (Carl Zeiss, Inc.).

Immunoblot. Immunoblot was performed with a monoclonal Twist-specific antibody (αTwiMab-1) (45) conjugated in-house to digoxygenin (Roche Diagnostics), anti–tubulin-α (DM1A; Calbiochem), anti-T-bet (4B10; Santa Cruz Biotechnology, Inc.), or anti-IκBα (Cell Signaling Technology), followed by incubation with horseradish peroxidase–coupled anti-digoxigenin FAB-fragments (Roche) or anti–mouse or anti–rabbit (Santa Cruz Biotechnology, Inc.) secondary antibodies. Individual bands were visualized with enhanced chemiluminescence (GE Healthcare) and the Intelligent Dark Box System LAS-3000 (Fujifilm).

Retroviral expression vectors and retroviral infection. The *twist1*-targeting shRNA vector was generated by amplification of the EF1α-promotor and GFP from pLVTHM, provided by D. Trono (Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland) followed ligation into pQCXIX (Clontech Laboratories, Inc.) with XbaI and EcoRV. The *twist1* target sequence corresponds to Twist-siRNA3 (5′-AAGCTGAGCAAGATTCAGACC-3′), as published by Yang et al. (3). A corresponding scrambled sequence was used as control. The DNA oligonucleotides were subcloned using HpaI and XhoI into pLL3.7, which was provided by L. Van Parijs (Massachusetts Institute of Technology, Cambridge, MA). The fragment containing the murine U6-promotor and the shRNA-encoding sequence was amplified by PCR, introducing an additional 5′-XhoI site and ligated into the SalI site of pQXIX-*gfp* located in the inactivated 3′-LTR. For retroviral overexpression the vector GFP-RV (46) was used, provided by K.M. Murphy (Howard Hughes Medical Institute, St. Louis, Missouri). Murine *t-bet* (cDNA generated from Th1-cells), murine *twist1* (IMAGE cDNA clone; AccessionID: BC033434-NCBI), and constitutively active IκBαM (pCMV-IκBαM; Clontech Laboratories, Inc.) were amplified, introducing BglII- and XhoI-compatible restriction sites and ligated into the vector upstream of the internal ribosome entry site-*gfp* cassette. Sequences of primers and oligonucleotides for shRNA expression are listed in Table S5. Retroviral stocks were obtained by calcium phosphate cotransfection of HEK293 cells with the retrovirus packaging plasmids pECO and pCGP. The medium was replaced after 4 h, and viral supernatants were collected 24–48 h later. Th cells were infected 40 h after activation by 60 min centrifugation at 700 g at 30°C with viral supernatant and 8 μg/ml polybrene, followed by replacement of the viral supernatant with the former culture supernatant.

Luciferase reporter assay. NF-κB activity was monitored using the pNF-κB-Luc vector (Clontech Laboratories, Inc.). Transfection efficiency was controlled by cotransfecting pRL-TK (Promega). Murine T cells were electroporated with the reporter constructs using Mouse T cell kit (AMAXA) and Nucleofector I (AMAXA). Luciferase activity was quantified with Dual Luciferase Assay kit (Promega) and a luminometer (Moonlight 3096; BD Biosciences).

Microarray experiments. Total RNA was extracted using Trizol reagent (Invitrogen), 10 μg was reverse-transcribed, followed by cDNA extraction with a PhaseLock gel (Eppendorf), and precipitated with ethanol and ammonium acetate. Biotinylated cRNA was transcribed with the MEGAscript high-yield transcription kit (Ambion), fragmented, and the hybridization cocktail was prepared according to Affymetrix protocols (15 μg fragmented biotin-labeled cRNA spiked with Eukaryotic Hybridization control). The

Published July 28, 2008

Murine Genome U74A version 2, and 430A version 2 GeneChip arrays (Affymetrix) were hybridized at 45°C for 16 h, stained with streptavidin-phycoerythrin using the Affymetrix GeneChip Fluidics Workstation 400, and scanned on a Hewlett-Packard Gene Array Scanner (MGU74Av2 arrays) or on an Affymetrix GeneChip Scanner 3000 (MG430Av2 arrays). Data were analyzed using the Microarray Suite 5.0 software (Affymetrix). Microarrays were globally normalized and scaled to a trimmed mean expression value of 200. All arrays were compared with each other, and a relational database was generated using Microsoft Access software, including the following parameters: expression heights, call for presence of transcripts, P value for presence or absence of transcripts, $\log_2$ value of fold change and 95% confidence intervals, call for the significance of differentially expression, and the P value for that call. For each transcript the significance of differential expression between the groups of arrays was calculated using strict Bonferroni-corrected Welch *t* tests. Significantly differentially expressed genes were filtered according to the following criteria: mean fold change ≥2 or ≥1.5; difference of means ≥200; P value ≤0.05; and immunoglobulin genes were excluded. Data are deposited at http://www.ncbi.nlm.nih.gov/geo/ (AccessionID SuperSeries: GSE11556).

Real-time PCR. Real-time PCR was performed as previously described (15). For normalization of murine and human cDNA the transcripts for the housekeeping genes hypoxanthine guanine phosphoribosyl transferase and ubiquitin ligase H5 (UbcH5) were quantified, respectively. Relative expression was calculated as follows: $E_t^{\Delta Cp \text{ target gene (reference - sample)}} / E_h^{\Delta Cp \text{ housekeeping gene (reference - sample)}}$, where Cp represents the crossing point and E represents the reaction efficiency, determined by serial dilution of DNA. Primer sequences are listed in Table S5 (available at http://www.jem.org/cgi/content/full/jem.20072468/DC1).

In silico genomic DNA analysis. The genomic sequences for the *twist1* locus of *Mus musculus* and *Homo sapiens* were obtained from UCSC Genome Bioinformatics (http://genome.ucsc.edu) and submitted to Matinspector analysis at http://www.genomatix.de/matinspector.html.

Statistics. Statistical significance in animal models (DTH and antigen-induced arthritis) was calculated using the two-tailed Mann–Whitney test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$).

Online supplemental material. Fig. S1 shows representative cytokine profiles of ex vivo–polarized Th1, Th2, and Th17 cells. Fig. S2 displays the result of ectopic T-bet expression on *twist1* mRNA induction, as well as an immunoblot to confirm T-bet overexpression. Fig. S3 shows cytokine data and mRNA profiles of cells cultured in the simultaneous presence of IL-12 and IL-4 compared with Th1 cells. The ChIP for acetylated and trimethylated histone 3 binding to the *twist1* promoter are shown in Fig. S4. The dot plots comparing cytokine expression in *twist1* overexpressing and control Th cells are shown in Fig. S5. Immunoblots confirming comparable protein expression levels of Twist1 and IκB-αM upon retroviral overexpression are shown in Fig. S6. Fig. S7 displays in vivo migration capacity of *twist1* overexpressing and control Th1 cells to remaining organs despite footpad and draining lymph nodes in the DTH response. Fig. S8 shows additional immunohistochemical stainings of the inflammatory response in murine arthritis. In Fig. S9, the effect of type I IFNs on *twist1* induction is shown. Genes differentially expressed in once- versus four-time–stimulated Th1 cells are shown in Table S1. Clinical characteristics of patients in the study are listed in Table S2. Genes differentially expressed upon ectopic *twist1* overexpression are listed in Table S3. Table S4 shows detailed results of the histological scoring in murine arthritis. Table S5 shows primer sequences. The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20072468/DC1.

We thank Simone Spieckermann for excellent technical assistance in immunohistochemistry experiments. We thank R. Brothwick and C. Raulfs for critical reading; S. Pade for collection of clinical samples; D. Trono for provision of the pLVTHM vector; L. Van Parijs for pLL3.7; and K.M. Murphy for GFP-RV.

This work was supported by the German Ministry of Education and Research (BMBF grant 1KW9913 and 0313068), within the National Genome Research Network (NGFN 1-2, BMBF grant 01GS0110, 01GS0160, 01GS0413), by the German Science Foundation (SFB 421, SFB 633) and by the European Community Grant MUGEN LSHG-CT-2005-005203. U. Niesner was a recipient of a doctoral fellowship from Boehringer Ingelheim Fonds.

The authors have no conflicting financial interests.

Submitted: 20 November 2007
Accepted: 16 June 2008

REFERENCES

1. Thisse, B., M. el Messal, and F. Perrin-Schmitt. 1987. The twist gene: isolation of a *Drosophila* zygotic gene necessary for the establishment of dorsoventral pattern. *Nucleic Acids Res.* 15:3439–3453.
2. Chen, Z.F., and R.R. Behringer. 1995. Twist is required in head mesenchyme for cranial neural tube morphogenesis. *Genes Dev.* 9:686–699.
3. Yang, J., S.A. Mani, J.L. Donaher, S. Ramaswamy, R.A. Itzykson, C. Come, P. Savagner, I. Gitelman, A. Richardson, and R.A. Weinberg. 2004. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell.* 117:927–939.
4. Sosic, D., J.A. Richardson, K. Yu, D.M. Ornitz, and E.N. Olson. 2003. Twist regulates cytokine gene expression through a negative feedback loop that represses NF-kappaB activity. *Cell.* 112:169–180.
5. Sharif, M.N., D. Sosic, C.V. Rothlin, E. Kelly, G. Lemke, E.N. Olson, and L.B. Ivashkiv. 2006. Twist mediates suppression of inflammation by type I IFNs and Axl. *J. Exp. Med.* 203:1891–1901.
6. Katz, J.D., C. Benoist, and D. Mathis. 1995. T helper cell subsets in insulin-dependent diabetes. *Science.* 268:1185–1188.
7. Iqbal, N., J.R. Oliver, F.H. Wagner, A.S. Lazenby, C.O. Elson, and C.T. Weaver. 2002. T helper 1 and T helper 2 cells are pathogenic in an antigen-specific model of colitis. *J. Exp. Med.* 195:71–84.
8. Maffia, P., J.M. Brewer, J.A. Gracie, A. Ianaro, B.P. Leung, P.J. Mitchell, K.M. Smith, I.B. McInnes, and P. Garside. 2004. Inducing experimental arthritis and breaking self-tolerance to joint-specific antigens with trackable, ovalbumin-specific T cells. *J. Immunol.* 173:151–156.
9. Rotondi, M., E. Lazzeri, P. Romagnani, and M. Serio. 2003. Role for interferon-gamma inducible chemokines in endocrine autoimmunity: an expanding field. *J. Endocrinol. Invest.* 26:177–180.
10. Morita, Y., M. Yamamura, M. Kawashima, S. Harada, K. Tsuji, K. Shibuya, K. Maruyama, and H. Makino. 1998. Flow cytometric single-cell analysis of cytokine production by CD4+ T cells in synovial tissue and peripheral blood from patients with rheumatoid arthritis. *Arthritis Rheum.* 41:1669–1676.
11. Fuss, I.J., M. Neurath, M. Boirivant, J.S. Klein, C. de la Motte, S.A. Strong, C. Fiocchi, and W. Strober. 1996. Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. *J. Immunol.* 157:1261–1270.
12. Skurkovich, B., and S. Skurkovich. 2006. Inhibition of IFN-gamma as a method of treatment of various autoimmune diseases, including skin diseases. *Ernst Schering Res. Found. Workshop.* 56:1–27.
13. Hommes, D.W., T.L. Mikhajlova, S. Stoinov, D. Stimac, B. Vucelic, J. Lonovics, M. Zakuciova, G. D'Haens, G. Van Assche, S. Ba, et al. 2006. Fontolizumab, a humanised anti-interferon gamma antibody, demonstrates safety and clinical activity in patients with moderate to severe Crohn's disease. *Gut.* 55:1131–1137.
14. van der Veen, R.C., T.A. Dietlin, and F.M. Hofman. 2003. Tissue expression of inducible nitric oxide synthase requires IFN-gamma production by infiltrating splenic T cells: more evidence for immunosuppression by nitric oxide. *J. Neuroimmunol.* 145:86–90.
15. Chang, H.D., C. Helbig, L. Tykocinski, S. Kreher, U. Niesner, and A. Radbruch. 2007. Expression of IL-10 in Th memory lymphocytes is conditional on IL-12 or IL-4, unless the IL-10 gene is imprinted by GATA-3. *Eur. J. Immunol.* 37:801–817.
16. Veldhoen, M., R.J. Hocking, C.J. Atkins, R.M. Locksley, and B. Stockinger. 2006. TGFbeta in the context of an inflammatory cytokine milieu

supports de novo differentiation of IL-17-producing T cells. *Immunity*. 24:179–189.
17. Harrington, L.E., R.D. Hatton, P.R. Mangan, H. Turner, T.L. Murphy, K.M. Murphy, and C.T. Weaver. 2005. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat. Immunol.* 6:1123–1132.
18. Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature*. 401:708–712.
19. Szabo, S.J., S.T. Kim, G.L. Costa, X. Zhang, C.G. Fathman, and L.H. Glimcher. 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell*. 100:655–669.
20. Sun, S.C., P.A. Ganchi, D.W. Ballard, and W.C. Greene. 1993. NF-kappa B controls expression of inhibitor I kappa B alpha: evidence for an inducible autoregulatory pathway. *Science*. 259:1912–1915.
21. Trevillyan, J.M., X.G. Chiou, Y.W. Chen, S.J. Ballaron, M.P. Sheets, M.L. Smith, P.E. Wiedeman, U. Warrior, J. Wilkins, E.J. Gubbins, et al. 2001. Potent inhibition of NFAT activation and T cell cytokine production by novel low molecular weight pyrazole compounds. *J. Biol. Chem.* 276:48118–48126.
22. Marienfeld, R., M. Neumann, S. Chuvpilo, C. Escher, B. Kneitz, A. Avots, A. Schimpl, and E. Serfling. 1997. Cyclosporin A interferes with the inducible degradation of NF-kappa B inhibitors, but not with the processing of p105/NF-kappa B1 in T cells. *Eur. J. Immunol.* 27:1601–1609.
23. Singer, G.G., A.C. Carrera, A. Marshak-Rothstein, C. Martinez, and A.K. Abbas. 1994. Apoptosis, Fas and systemic autoimmunity: the MRL-lpr/lpr model. *Curr. Opin. Immunol.* 6:913–920.
24. Cohen, P.L., and R.A. Eisenberg. 1991. Lpr and gld: single gene models of systemic autoimmunity and lymphoproliferative disease. *Annu. Rev. Immunol.* 9:243–269.
25. Mixter, P.F., J.Q. Russell, G.J. Morrissette, C. Charland, D. Aleman-Hoey, and R.C. Budd. 1999. A model for the origin of TCR-alpha-beta+ CD4-CD8- B220+ cells based on high affinity TCR signals. *J. Immunol.* 162:5747–5756.
26. De Jong, R., M. Brouwer, B. Hooibrink, T. Van der Pouw-Kraan, F. Miedema, and R.A. Van Lier. 1992. The CD27- subset of peripheral blood memory CD4+ lymphocytes contains functionally differentiated T lymphocytes that develop by persistent antigenic stimulation in vivo. *Eur. J. Immunol.* 22:993–999.
27. Fritsch, R.D., X. Shen, G.P. Sims, K.S. Hathcock, R.J. Hodes, and P.E. Lipsky. 2005. Stepwise differentiation of CD4 memory T cells defined by expression of CCR7 and CD27. *J. Immunol.* 175:6489–6497.
28. Bonecchi, R., G. Bianchi, P.P. Bordignon, D. D'Ambrosio, R. Lang, A. Borsatti, S. Sozzani, P. Allavena, P.A. Gray, A. Mantovani, and F. Sinigaglia. 1998. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J. Exp. Med.* 187:129–134.
29. Nagata, K., K. Tanaka, K. Ogawa, K. Kemmotsu, T. Imai, O. Yoshie, H. Abe, K. Tada, M. Nakamura, K. Sugamura, and S. Takano. 1999. Selective expression of a novel surface molecule by human Th2 cells in vivo. *J. Immunol.* 162:1278–1286.
30. Wan, Y.Y., and J. DeGregori. 2003. The survival of antigen-stimulated T cells requires NFkappaB-mediated inhibition of p73 expression. *Immunity*. 18:331–342.
31. Schmidt-Supprian, M., G. Courtois, J. Tian, A.J. Coyle, A. Israel, K. Rajewsky, and M. Pasparakis. 2003. Mature T cells depend on signaling through the IKK complex. *Immunity*. 19:377–389.
32. Fong, T.A., and T.R. Mosmann. 1989. The role of IFN-gamma in delayed-type hypersensitivity mediated by Th1 clones. *J. Immunol.* 143: 2887–2893.
33. Annunziato, F., L. Cosmi, V. Santarlasci, L. Maggi, F. Liotta, B. Mazzinghi, E. Parente, L. Filì, S. Ferri, F. Frosali, et al. 2007. Phenotypic and functional features of human Th17 cells. *J. Exp. Med.* 204: 1849–1861.
34. Murre, C., P.S. McCaw, and D. Baltimore. 1989. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. *Cell*. 56:777–783.
35. Choy, E.H., C. Pitzalis, A. Cauli, J.A. Bijl, A. Schantz, J. Woody, G.H. Kingsley, and G.S. Panayi. 1996. Percentage of anti-CD4 monoclonal antibody-coated lymphocytes in the rheumatoid joint is associated with clinical improvement. Implications for the development of immunotherapeutic dosing regimens. *Arthritis Rheum.* 39:52–56.
36. Emmrich, F., G. Horneff, W. Becker, W. Luke, A. Potocnik, U. Kanzy, J.R. Kalden, and G. Burmester. 1991. An anti-CD4 antibody for treatment of chronic inflammatory arthritis. *Agents Actions Suppl.* 32:165–170.
37. Horneff, G., G.R. Burmester, F. Emmrich, and J.R. Kalden. 1991. Treatment of rheumatoid arthritis with an anti-CD4 monoclonal antibody. *Arthritis Rheum.* 34:129–140.
38. Nepom, G.T. 2002. Therapy of autoimmune diseases: clinical trials and new biologics. *Curr. Opin. Immunol.* 14:812–815.
39. Panaccione, R., J.G. Ferraz, and P. Beck. 2005. Advances in medical therapy of inflammatory bowel disease. *Curr. Opin. Pharmacol.* 5: 566–572.
40. Baumgart, D.C., and A.U. Dignass. 2004. Current biological therapies for inflammatory bowel disease. *Curr. Pharm. Des.* 10:4127–4147.
41. Truelove, S.C., and L.J. Witts. 1955. Cortisone in ulcerative colitis; final report on a therapeutic trial. *BMJ*. 2:1041–1048.
42. Harvey, R.F., and J.M. Bradshaw. 1980. A simple index of Crohn's-disease activity. *Lancet*. 1:514.
43. Feuerer, M., K. Eulenburg, C. Loddenkemper, A. Hamann, and J. Huehn. 2006. Self-limitation of Th1-mediated inflammation by IFN-gamma. *J. Immunol.* 176:2857–2863.
44. Siegmund, K., M. Feuerer, C. Siewert, S. Ghani, U. Haubold, A. Dankof, V. Krenn, M.P. Schon, A. Scheffold, J.B. Lowe, A. Hamann, U. Syrbe, and J. Huehn. 2005. Migration matters: regulatory T-cell compartmentalization determines suppressive activity in vivo. *Blood*. 106:3097–3104.
45. Gitelman, I. 1997. Twist protein in mouse embryogenesis. *Dev. Biol.* 189:205–214.
46. Afkarian, M., J.R. Sedy, J. Yang, N.G. Jacobson, N. Cereb, S.Y. Yang, T.L. Murphy, and K.M. Murphy. 2002. T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells. *Nat. Immunol.* 3:549–557.

3.6 Twist1 fördert das Überleben von Th1-Zellen über die microRNA miR-148a

Haftmann C, Stittrich AB, Zimmermann J, Fang Z, Hradilkova K, Bardua M, Westendorf K, Heinz GA, Riedel R, Siede J, Lehmann K, Weinberger EE, Zimmel D, Lauer U, Häupl T, Sieper J, Backhaus M, Neumann C, Hoffmann U, Porstner M, Chen W, Grün JR, Baumgrass R, Matz M, Löhning M, Scheffold A, Wittmann J, **Chang HD**, Rajewsky N, Jäck HM, Radbruch A, Mashreghi MF. 2015. miR-148a is upregulated by Twist1 and T-bet and promotes Th1-cell survival by regulating the proapoptotic gene Bim. *Eur J Immunol.* 45(4):1192-205.

Die Inhibition von miRNA-Funktionen in Th- Zellen durch das Ausschalten von Dicer hat gezeigt, dass miRNAs eine wichtige Rolle bei der Differenzierung und den Funktionen von Th-Zellen spielen [98]. Analog zum Genexpressionsvergleich zwischen ein- und mehrfach restimulierten Th-Zellen haben wir daher die differentielle Expression von miRNAs untersucht. Unter den differentiell exprimierten miRNAs war miR-148a selektiv in mehrfach restimulierten Th1-Zellen hochreguliert. Unsere Analysen haben ergeben, dass der proapoptotische Faktor Bim (kodiert durch das Gen *Bcl2l11*) durch miR-148a negativ reguliert wird. Die Inhibition von miR-148a in Th1-Zellen durch spezifische Antagomire resultierte in einer Hochregulation der Bim Expression und in einer höheren Apoptoserate unter den Th-Zellen. Antagomire sind modifizierte Oligonukleotide, die membrangängig sind und die ihre Ziel-microRNA spezifisch binden und dadurch ihre Funktion unterbinden. Da die miR-148a ein ähnliches Expressionsprofil zeigte wie Twist1 und wir auch in vorangegangenen Analysen zeigen konnten, dass die Inhibition von Twist1 mittels shRNA oder genetischem Knock-out auch in einer Erhöhung der Bim-Expression resultierte, haben wir untersucht ob Twist1 eine Rolle bei der miR-148a-Regulation spielt. Tatsächlich war in Th-Zellen, in denen die Twist1-Expression inhibiert wurde, auch die Expression der miR-148a reduziert. Durch ChIP-Analyse konnten wir nachweisen, dass Twist1 direkt an das miR-148a-Gen bindet und somit die Expression direkt reguliert.
Somit konnten wir zeigen, dass Twist1 nicht nur eine Rolle bei der Selbstregulation von Th1-Entzündungen spielt, sondern auch zum Überleben der Th1-Zellen durch die Induktion von miR-148a beiträgt. Twist1 und miR-148a könnten somit zur Chronifizierung von Th1-vermittelten Entzündungen beitragen, indem sie die Persistenz der Th1-Zellen im entzündeten Gewebe fördern.

 DOI: 10.1002/eji.201444633 Eur. J. Immunol. 2015. 45: 1192–1205

European Journal of Immunology

miR-148a is upregulated by Twist1 and T-bet and promotes Th1-cell survival by regulating the proapoptotic gene Bim

Claudia Haftmann[*1], *Anna-Barbara Stittrich*[*1,2], *Jakob Zimmermann*[1], *Zhuo Fang*[1,3], *Kristyna Hradilkova*[1], *Markus Bardua*[1], *Kerstin Westendorf*[1], *Gitta A. Heinz*[1,4], *René Riedel*[1], *Julia Siede*[1,5], *Katrin Lehmann*[1], *Esther E. Weinberger*[1,5], *David Zimmel*[1,5], *Uta Lauer*[5], *Thomas Häupl*[5], *Joachim Sieper*[5], *Marina Backhaus*[5], *Christian Neumann*[1], *Ute Hoffmann*[1,5], *Martina Porstner*[6], *Wei Chen*[7], *Joachim R Grün*[1], *Ria Baumgrass*[1], *Mareen Matz*[8], *Max Löhning*[1,5], *Alexander Scheffold*[5], *Jürgen Wittmann*[6], *Hyun-Dong Chang*[1], *Nikolaus Rajewsky*[3], *Hans-Martin Jäck*[6], *Andreas Radbruch*[*1] and *Mir-Farzin Mashreghi*[*1]

[1] Deutsches Rheuma-Forschungszentrum Berlin (DRFZ), an institute of the Leibniz AssociationBerlin, Berlin, Germany
[2] Institute for Systems Biology, Seattle, WA, USA
[3] Laboratory of Systems Biology of Gene Regulatory Elements, Max-Delbrück-Center for Molecular Medicine, Berlin, Germany
[4] Institute of Molecular Immunology, Helmholtz Zentrum München, Munich, Germany
[5] Department of Rheumatology and Clinical Immunology, Charité-Universitätsmedizin Berlin, Berlin, Germany
[6] Division of Molecular Immunology, Friedrich-Alexander University Erlangen Nürnberg, Erlangen, Germany
[7] Laboratory of Novel sequencing technology, Function and Medical Genomics, Max-Delbrück-Center for Molecular Medicine, Berlin, Germany
[8] Department of Nephrology, Charité-Universitätsmedizin, Berlin, Germany

Repeatedly activated T helper 1 (Th1) cells present during chronic inflammation can efficiently adapt to the inflammatory milieu, for example, by expressing the transcription factor Twist1, which limits the immunopathology caused by Th1 cells. Here, we show that in repeatedly activated murine Th1 cells, Twist1 and T-bet induce expression of microRNA-148a (miR-148a). miR-148a regulates expression of the proapoptotic gene *Bim*, resulting in a decreased *Bim/Bcl2* ratio. Inhibition of miR-148a by antagomirs in repeatedly activated Th1 cells increases the expression of Bim, leading to enhanced apoptosis. Knockdown of *Bim* expression by siRNA in miR-148a antagomir-treated cells restores viability of the Th1 cells, demonstrating that miR-148a controls survival by regulating *Bim* expression. Thus, Twist1 and T-bet not only control the differentiation and function of Th1 cells, but also their persistence in chronic inflammation.

Keywords: Bim · miR-148a · T-bet · Th1 · Twist1

See accompanying article by Porstner et al.

Additional supporting information may be found in the online version of this article at the publisher's web-site

Correspondence: Dr. Mir-Farzin Mashreghi
e-mail: mashreghi@drfz.de

*These authors contributed equally to this work.

Introduction

Immune responses against intracellular microorganisms are mediated by T helper type 1 (Th1) cells, which produce interferon (IFN)-γ as their hallmark cytokine [1]. The differentiation of naive $CD4^+$ cells into Th1 cells is dynamically regulated and includes signals from (i) the T-cell receptor (TCR), (ii) the IFN-γ receptor/ signal transducer and activator of transcription (STAT)-1 and (iii) the interleukin (IL)-12 receptor/STAT4 which collectively lead to the induction and further amplification of the Th1 master transcription factor T-bet [2]. Besides their protective role in the clearance of infection, Th1 cells are involved in the initiation and maintenance of inflammatory diseases and disease models such as inflammatory bowel disease [3], OVA-induced arthritis [4], and experimental autoimmune encephalomyelitis (EAE) [5]. Repeatedly activated Th1 cells accumulate in inflamed tissues [6–8] and persist, despite therapeutic immunosuppression, in patients suffering from chronic inflammatory diseases [9]. These cells express the transcription factor Twist1, whose expression increases upon repeated restimulation of Th1 cells and limits immunopathology in chronic inflammation [9]. However, it has remained elusive how the persistence of Th1 cells in inflamed tissue is mediated.

The B-cell lymphoma 2 (Bcl-2) family proteins control the contraction of activated T cell populations after an adaptive immune response and are composed of proapoptotic and antiapoptotic proteins [10–12]. The antiapoptotic protein Bcl-2 stabilizes the mitochondrial outer membrane and inhibits its permeabilization. In the contraction phase of an adaptive immune response, withdrawal of IL-2 or IL-7 leads to the induction and activation of Bim, which interacts with and antagonizes the function of Bcl-2 and promotes the intrinsic apoptosis pathway in activated T cells [10, 13]. Excess of Bim destabilizes the mitochondrial outer membrane, leading to activation of caspase 9 and in consequence to induction of apoptosis [14]. Thus, the regulation of Bcl-2 and Bim expression is critical for the survival of T cells [15, 16].

Here we show that in repeatedly activated Th1 cells, Bim expression is downregulated posttranscriptionally by miR-148a. Expression of miR-148a is induced by the transcription factors T-bet and Twist1, thus is selective for repeatedly activated Th1 cells. This mechanism promotes persistence of antigen-specific Th1 cells in long-lasting, chronic immune reactions, and identifies Twist1 as a critical regulator of chronicity in inflammation.

Results

miR-148a is upregulated in repeatedly activated Th1 cells

To address miR-148a expression in Th cells, murine naive ($CD4^+CD62L^{hi}$) Th cells were activated in vitro under polarizing conditions for the generation of effector T helper type I (Th1 once), type II (Th2 once), and IL-17-producing (Th17 once) cells. Mimicking Th cells involved in chronic inflammation with a history of repeated restimulation by persistent (auto-) antigens, we repeatedly (4x) activated cells of the three different Th cell subsets. Quality of differentiation was controlled by intracellular cytokine staining for IFN-γ, IL-4, and IL-17 (Supporting Information Fig.1A–C). miR-148a was expressed in naive and once activated Th1, Th2, and Th17 and significantly upregulated up to 30-fold in repeatedly activated Th1 (Th1 rep.), but not in Th2 (Th2 rep.) or Th17 cells (Th17 rep.; 1A). Already after one round of activation, all Th1 cells uniformly expressed T-bet, qualifying them as Th1 cells [17, 18] (Supporting Information Fig. 2A). Of those Th1 cells, those actually secreting IFN-γ in the restimulation and those that did not, showed the same level of miR-148a expression, and significantly less than Th1 rep. (Supporting Information Fig. 2B). miR-148a is a member of a miRNA family with miR-148b and miR-152 sharing the same seed region and potentially targeting the same genes (http://www.mirbase.org; [19]). Expression of miR-148b was sevenfold and expression of miR-152 30-fold lower than miR-148a expression in repeatedly activated Th1 cells (Fig. 1B). Both miR-148b and miR-152 were not differentially regulated between once and repeatedly activated Th1 cells (Fig. 1B). To address miR-148a expression in in vivo-differentiated Th1 cells, SMARTA1-TCR transgenic (SM TCRtg) $Thy1.1^+$ naive $CD4^+$ Th cells were transferred into nonlymphopenic C57BL/6 mice and infected with 200 pfu of lymphocytic choriomeningitis virus (LCMV) strain WE on day two after transfer. Seven days after infection, SM TCRtg $Thy1.1^+$ $CD4^+$ Th cells uniformly expressed T-bet (Fig. 1C and D) and consisted of 87% of IFN-γ producers (Supporting Information Fig. 2C). Notably, these Th1 cells expressed ninefold and 48-fold higher miR-148a levels compared to naive host $CD4^+$ T cells on day 5 and day 7 after viral infection, respectively, (Fig. 1E). These results show that miR-148a is exclusively expressed in repeatedly activated Th1 cells in vitro and Th1 cells differentiated after LCMV infection in vivo.

miR-148a targets the proapoptotic gene *Bim*

Candidate miR-148a targets in repeatedly activated Th1 cells were identified by target screens with PicTar (http://pictar.mdc-berlin.de/; [20]) and TargetScan (http://www.targetscan.org/; [21]), in combination with global transcriptome data of once versus repeatedly activated Th1 cells (Niesner et al. [9] and Supporting Information Table 1). Genome wide, 581 genes were identified as potential targets of miR-148a. Of these, 361 were expressed in Th1 cells and 130 genes were differentially expressed with a foldchange ≥ 1.4 in once versus repeatedly activated Th1 cells with 61 genes being down- and 69 genes being up-regulated in repeatedly activated Th1 cells. Among the predicted miR-148a targets was the proapoptotic gene *Bim*. Bim suited the hypothesis of a miR-148a mediated Th1 specific survival mechanism, which was raised from the observation that Th cells from inflamed tissues of autoimmune patients persist despite immunosuppressive therapy. Expression levels of *Bim* mRNA and protein were reduced twofold in repeatedly activated Th1 cells as compared to once activated Th1 cells (Supporting Information Table 1 and

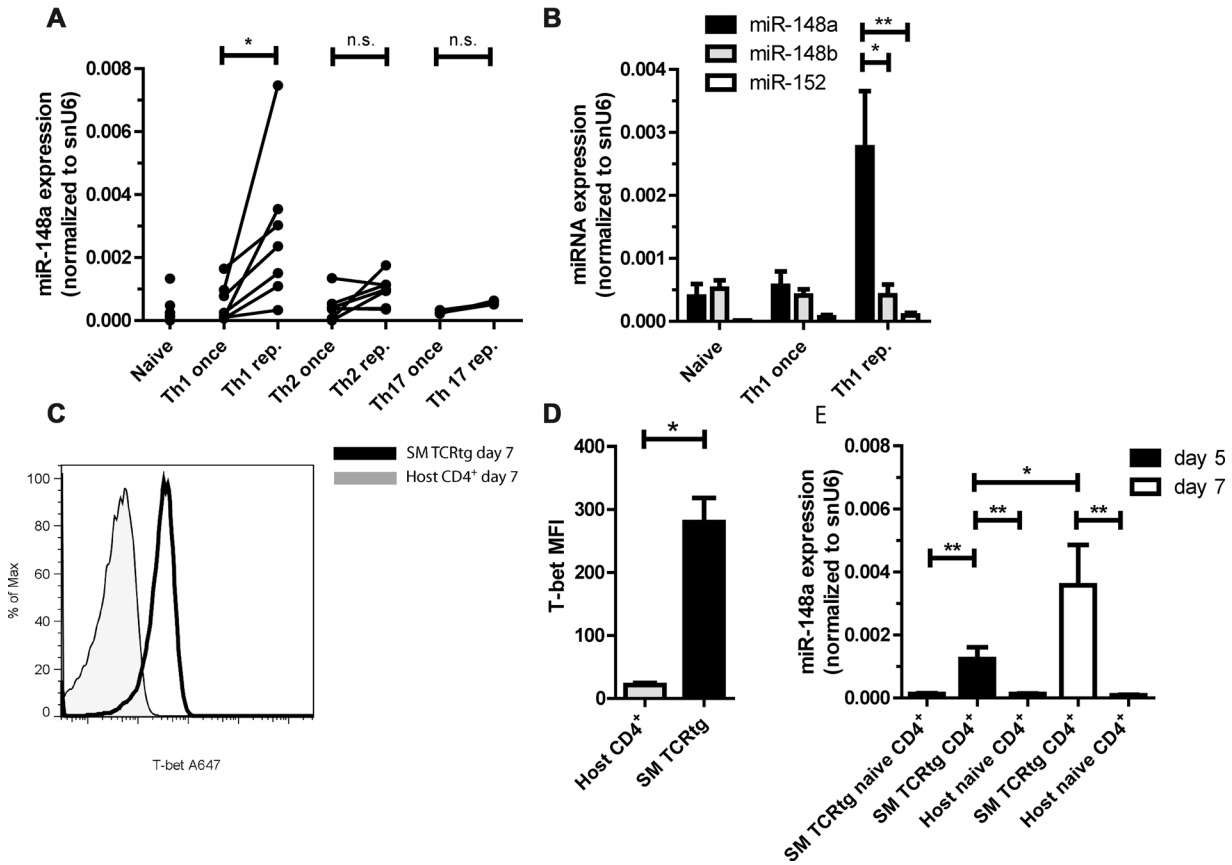


Figure 1. miR-148a is upregulated in repeatedly activated Th1 cells (A) Quantitative RT-PCR (qRT-PCR) of miR-148a expression in once (on day 6) and repeatedly (6 day intervals; three rounds of restimulation) activated Th1 cells (independent experiments, $n = 7$), Th2 cells ($n = 7$), Th17 cells ($n = 3$), ex vivo-isolated naive Th cells ($n = 6$), normalized to small nuclear RNA U6 (snU6). Each data point represents an independent experiment. Data are shown as mean + SEM pooled from three to seven independent experiments. Wilcoxon-Test for paired data, $^{*}p \leq 0.05$. (B) Expression of miRNA family members miR-148a ($n = 7$, same cohort as in (A)) miR-148b (independent experiments, $n = 4$) and miR-152 (independent experiments, $n = 4$) in once and repeatedly activate d Th1 cells normalized to snU6, assessed by qRT-PCR. Each data point represents an independent experiment. Data are shown as mean ± SEM pooled from four to seven independent experiments. Mann–Whitney test for unpaired data, $^{*}p \leq 0.05$, $^{**}p \leq 0.005$. (C) T-bet protein expression in SM TCRtg CD4^{+} T cells on day 7 after LCMV infection was assessed by intracellular staining and flow cytometry. A single histogram from an experiment performed with five samples is shown, representative of three independent experiments performed. For gating strategies see Supporting Information Fig. 7A. (D) Statistical evaluation of MFIs of T-bet as determined in (C). Data are shown as mean + SEM of $n = 5$ samples from a single experiment representative of three experiments. Mann–Whitney test for unpaired data, $^{*}p \leq 0.05$. (E) qRT-PCR evaluation of miR-148a expression on day 5 and 7 of LCMV infection. Data are shown as mean ± SEM, depicted is one experiment with $n = 5$, representative of two independent experiments. Mann–Whitney test for unpaired data, $^{*}p \leq 0.05$, $^{**}p \leq 0.005$.

Fig. 2A and B). Ectopic overexpression of miR-148a in activated Th1 cells decreased levels of *Bim* mRNA and Bim protein by 50% (Fig. 2C–E). The two predicted miR-148a binding sites (bs) in the *Bim* 3′-UTR were validated in reporter assays. The *Bim* 3′-UTR was cloned downstream of a human CD4 reporter gene (hCD4) [22] (Supporting Information Fig. 3). Reporter gene expression (MFI of hCD4) was reduced by 30% in activated Th1 cells (d5) in the presence of a miR-148a overexpression vector (Fig. 2F). When both bs were destroyed by mutation (Supporting Information Fig. 3), this suppression was abrogated (Fig. 2F). By treating repeatedly activated Th1 cells with specific antagomirs [23], the inhibition of Bim expression by endogenous miR-148a was demonstrated. Antagomir-148a reduced miR-148a expression levels up to 98%, as compared to cells treated with a scrambled control antagomir, and increased *Bim* mRNA 1.8-fold while *Bcl2* expression remained unchanged (data not shown). On the protein level, Bim expression, as measured by intracellular immunofluorescence, increased 1.6-fold in repeatedly activated Th1 cells treated with antagomir-148a compared to cells treated with the scrambled antagomir (Fig. 2G and H). Bcl-2 protein expression remained similar in antagomir-148a treated cells (Fig. 2G and H and Supporting Information Fig.4A) leading to a significant shift in the ratio of Bim to Bcl2 expression in favor of Bim (Fig. 2H).

miR-148a has been reported to target other apoptosis regulators, for example, phosphatase and tensin homolog (Pten). The 3′-UTR of Pten contains conserved bs for miR-148a and a downregulation of Pten by miR-148a has been shown in hepatocytes [24]. In once activated Th1 cells, ectopic miR-148a overexpression downregulated the expression of a reporter construct containing the *Pten* 3′ -UTR depending on the presence of the miR-148a bs ($p < 0.05$; Fig. 3A) and *Pten* mRNA, the latter however, with low significance ($p = 0.125$; Fig. 3B). In contrast, inhibition of

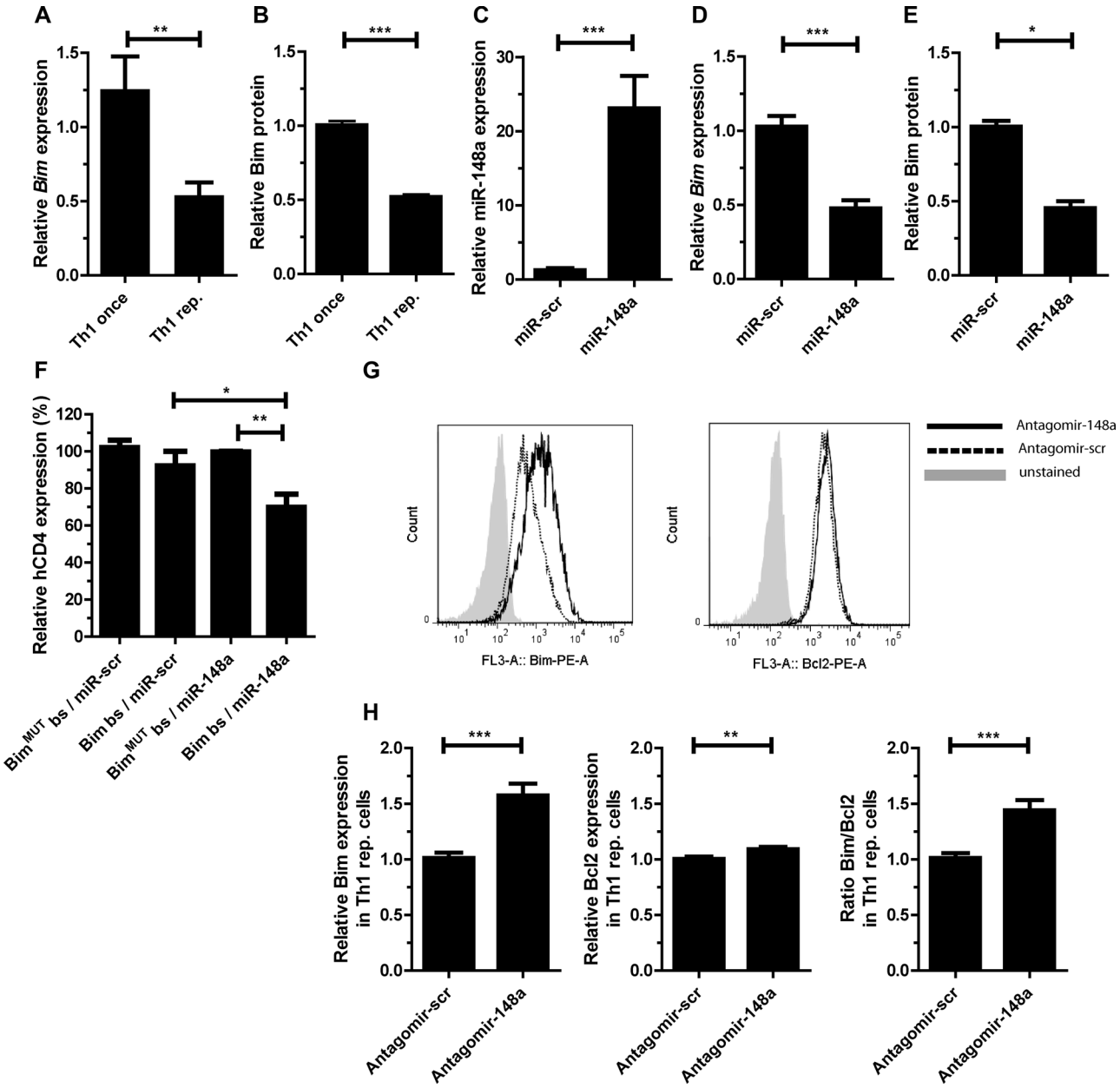


Figure 2. miR-148a targets Bim in repeatedly activated Th1 cells. (A) *Bim* mRNA expression in once and repeatedly activated Th1 cells was assessed by qRT-PCR, normalized to HPRT and presented relative to values obtained with once-activated Th1 cells. Data are shown as mean ± SEM, $n = 1$, each pooled from four independent experiments. (B) Bim protein expression in once and repeatedly activated Th1 cells assessed by intracellular protein staining and flow cytometry, presented as MFI of Bim, relative to once-activated Th1 cells. Data are shown as mean ± SEM, $n = 1$, each pooled from three independent experiments. For gating strategies see Supporting Information Fig. 7B. (C and D) Overexpression of miR-148a and a scrambled control (miR-scr) in $CD4^+$ T cells assessed by qRT-PCR, analyzed on day 5 postactivation. miR-148a and *Bim* expression was normalized to snU6 or HPRT and presented relative to values obtained with miR-scr. Data are shown as mean ± SEM, $n = 1$, each pooled from six (miR148a) or three (*Bim*) independent experiments. (E) Bim expression was validated by immunoblotting and represented relative to values obtained with miR-scr. Data are shown as mean ± SEM, $n = 1$, each pooled from two (miR-scr) or three (miR-148a) independent experiments. (F) Reporter gene expression in activated Th1 cells cotransduced with *Bim*3′-UTR reporter vector containing miR-148a bs (Bim bs) or mutated bs for the miR-148a (Bim^{MUT}bs) and an overexpression vector for miR-148a (miR-148a) or a scrambled overexpression vector (miR-scr), assessed by flow cytometry of the MFI of human CD4 on day 5 after activation and presented relative to values obtained for Bim^{MUT}bs/miR-148a. Data are shown as mean ± SEM, $n = 1$, each pooled from four independent experiments. For gating strategies see Supporting Information Fig. 7C. (G) Representative intracellular protein staining of Bim and Bcl2 in repeatedly activated Th1 cells after treatment with antagomir-148a or scrambled control (antagomir-scr) on day 3 postrestimulation with αCD3/αCD28, assessed by flow cytometry. Data shown are from one experiment representative of two independent experiments. For gating strategies see Supporting Information Fig. 7B. (H) Statistical evaluation of MFIs of Bim, Bcl2, and the ratio Bim/Bcl2 after treatment with antagomir-148a presented relative to values obtained with antagomir-scr. Data are shown as mean ± SEM, $n = 5$ pooled from two independent experiments. (A–F and H) Mann–Whitney test for unpaired data, $^*p \le 0.05$, $^{**}p \le 0.005$, $^{***}p \le 0.001$.

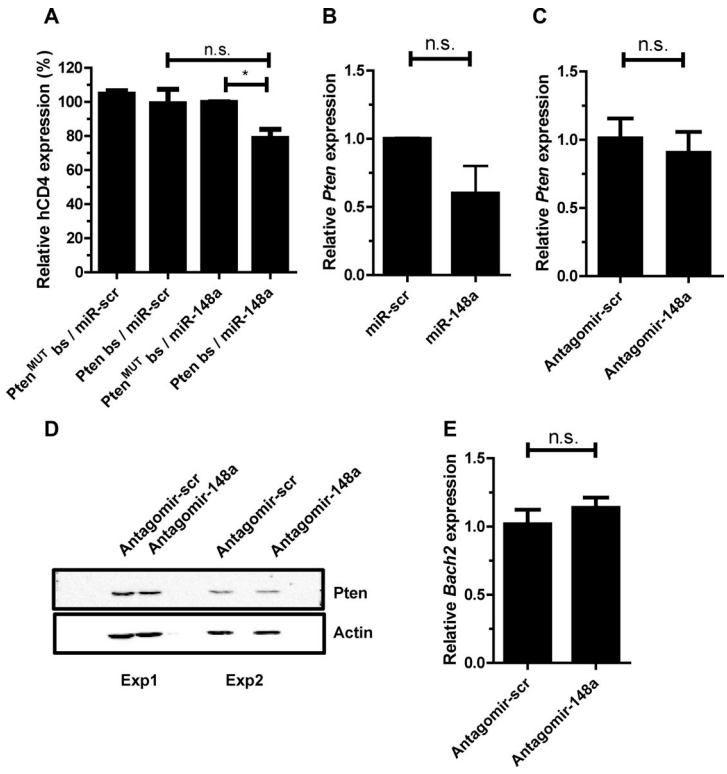


Figure 3. Mir-148a does no target Pten in repeatedly activated Th1 cells. (A) Reporter gene expression in activated Th1 cells cotransduced with *Pten*3′-UTR reporter vector containing miR-148a bs (Pten bs) or mutated bs for the miR-148a ($Pten^{MUT}$bs) and an overexpression vector for miR-148a (miR-148a) or a scrambled overexpression vector (miR-scr), assessed by flow cytometry. MFI of human CD4 on day 5 after activation are presented relative to values obtained for $Pten^{MUT}$bs/miR-148a. Data are shown as mean ± SEM, $n = 4$, pooled from two independent experiments. Mann–Whitney test for unpaired data, one-tailed, $^*p \leq 0.05$, n.s. $p = 0.125$. For gating strategies see Supporting Information Fig. 7C. (B) Overexpression of miR-148a and miR-scr in $CD4^+$ T cells, analyzed on day 5 postactivation. *Pten* expression was normalized to HPRT and are presented relative to values obtained with miR-scr. Data are shown as mean ± SEM, $n = 1$ each pooled from three independent experiments. Mann–Whitney test for unpaired data, n.s. $p = 0.125$. (C) *Pten* expression of repeatedly activated Th1 cells after antagomir treatment with antagomir-148a or antagomir-scr on day 3 post restimulation with αCD3/αCD28, normalized to HPRT and presented relative to values obtained with antagomir-scr. Data are shown as mean ± SEM, $n = 1$, each pooled from three independent experiments. (D) Pten expression in repeatedly activated Th1 cells after treatment with antagomir-148a or antagomir-scr on day 3 postrestimulation with αCD3/αCD28 validated by immunoblotting data from two independent experiments are shown. (E) *Bach2* expression in repeatedly activated Th1 cells after antagomir treatment, assessed by qRT-PCR, normalized to HPRT and presented relative to values obtained for antagomir-scr. Data are shown as mean ± SEM, $n = 3$, from one experiment representative of two independent experiments. Mann–Whitney test for unpaired data, one-tailed.

endogenous miR-148a expression levels in repeatedly activated Th1 cells, by antagomir-148a, did not change expression of endogenous Pten mRNA or protein (Fig. 3C and D). Another potential target is Broad-complex-Tramtrack-Bric-a-Brac and Cap'n'collar homology 1 bZip transcription factor 2 (Bach2) which is differentially expressed between once and repeatedly activated Th1 cells (Supporting Information Table 1). However, inhibition of endogenous miR-148a expression in repeatedly activated Th1 cells by antagomirs did not enhance expression of Bach2 (Fig. 3E).

Inhibition of miR-148a increases apoptosis of repeatedly activated Th1 cells after reactivation

It needed to be investigated whether the increase of Bim after miR-148a inhibition impacted on the survival of repeatedly activated Th1 cells. Treatment with antagomir-148a significantly decreased the numbers of repeatedly activated Th1 cells to 50 and 70% compared to control-treated cells on day 3 and 4 after restimulation, respectively (Fig. 4A). This was due to an increased apoptosis rate and not due to reduced proliferation (Supporting Information Fig. 4B). On day 3, antagomir-148a treated cells showed 30% more apoptotic cells after reactivation than controls (Fig. 4B and C). In repeatedly activated Th2 and Th17 cells antagomir mediated inhibition of miR-148a did not result in reduced numbers of viable cells (Supporting Information Fig. 5A–D), probably because of unchanged apoptosis rates (Supporting Information Fig. 5–E) and *Bim/Bcl2* ratio (Supporting Information Fig. 5–F). With respect to regulation of apoptosis in repeatedly activated Th1 cells, *Bim* is a main target of miR-148a in repeatedly activated Th1 cells, since knocking down *Bim* expression with a specific siRNA (siBim) in antagomir-148a treated cells restored their viability. In such cells, *Bim* expression was restored to levels observed in cells treated with a scrambled antagomir and a control siRNA (siScr) not targeting Bim (Fig. 4D–E). The numbers of viable repeatedly activated Th1 cells were reconstituted by 50% (Fig. 4F). Together, these results demonstrate that the survival mediated by miR-148a targeting Bim is unique in repeatedly activated Th1 cells.

Expression of miR-148a in Th1 cells is induced by T-bet and Twist1

As expression of miR-148a is upregulated selectively in repeatedly activated Th1 cells (Fig. 1A) it is likely that the expression of the miRNA is regulated by the Th1 specific transcription factors T-bet and Twist1. In repeatedly activated Th1 cells we observed a twofold induction of expression of the Th1 master transcription factor *Tbx21* (gene encoding for T-bet), as compared to once activated T cells (Fig. 5A). When activated under Th1-polarizing conditions, *Tbx21*-deficient $CD4^+$ T cells showed a four- to six-fold reduced induction of miR-148a expression (Fig. 5B). It is unlikely that T-bet directly induces miR-148a expression, since it has been shown that T-bet does not bind upstream of the miR-148a locus [25]. Ectopic overexpression of T-bet induced miR-148a expression 1.4-fold ($p = 0.091$) compared to an empty retroviral control vector. In accordance, overexpression of the dominant-negative

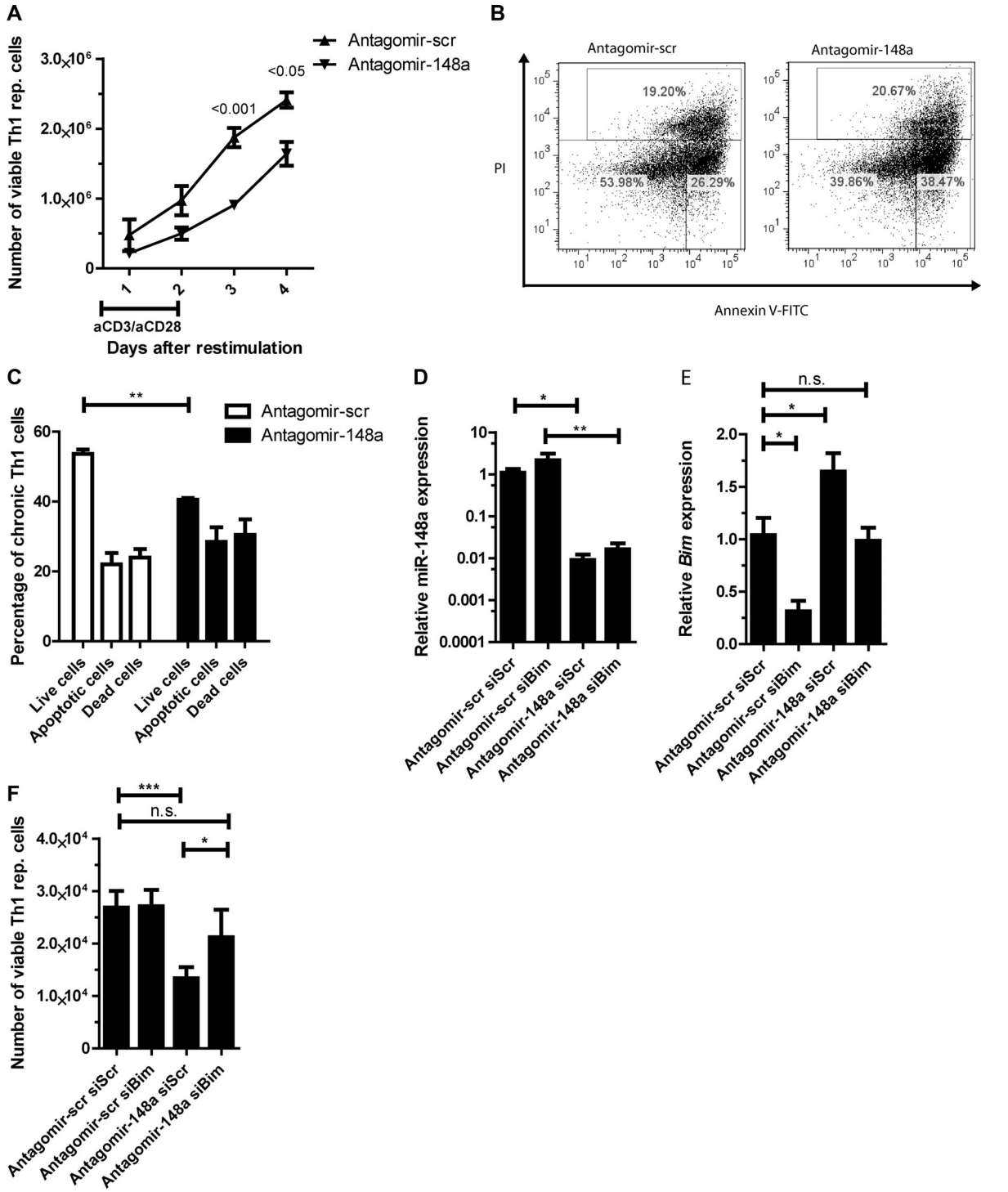


Figure 4. Inhibition of miR-148a results in increased apoptosis of repeatedly activated Th1 cells after reactivation. (A) The numbers of viable repeatedly activated Th1 cells after antagomir treatment and restimulation with αCD3/αCD28, was assessed by flow cytometry with $n = 5$ for day 1, 2, and 3, $n = 3$ for day 4. Data are shown as mean ± SEM, $n = 5$ (day 1–3) or $n = 3$ (day 4), pooled from two independent experiments. Two-way ANOVA with Bonferroni correction. For gating strategies see Supporting Information Fig. 7D. (B) Representative annexinV/PI staining of repeatedly activated Th1 cells and (C) frequencies of live cells (annexinV^-PI^-), apoptotic cells (annexinV^+PI^-), and dead cells (annexinV^+PI^+) after antagomir treatment assessed by annexinV/PI staining followed by flow cytometry on day 3 postrestimulation with αCD3/αCD28. Data are shown as mean + SEM, $n = 6$/group, pooled from four independent experiments. For gating strategies see Supporting Information Fig. 7E. (D–F) Repeatedly activated Th1 cells were treated with antagomir-148a / antagomir-scr and siRNA against *Bim* (siBim) or a nontargeting control (siScr) prior to restimulation with αCD3/αCD28. (D and E) qRT-PCR was used to assess (D) miR-148a and (E) *Bim* expression, which was normalized to snU6 or HPRT and presented relative to values obtained with antagomir-scr and siScr. (F) The number of viable Th1 cells was determined by flow cytometry. Data are shown as mean ± SEM, $n = 7$/group, pooled from three independent experiments. (C–F) Mann–Whitney test for unpaired data, $^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$).

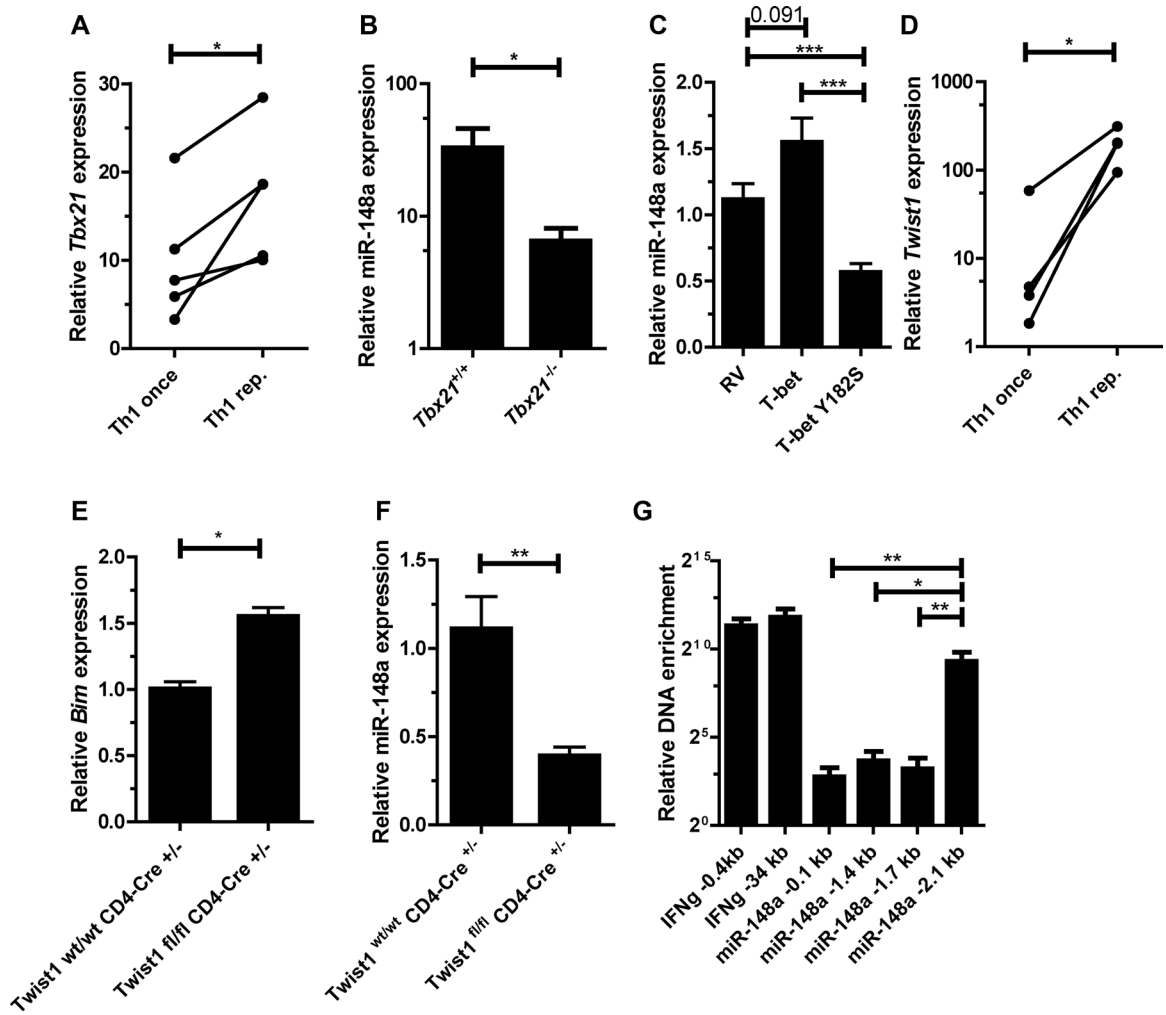


Figure 5. Expression of miR-148a in Th1 cells is induced by T-bet and Twist1. (A) *Tbx21* expression in once and repeatedly activated Th1 cells was assessed by qRT-PCR, normalized to HPRT and presented relative to values obtained for naive Th cells. Each data point represents an individual experiment performed with $n = 1$. Wilcoxon-test for paired data, $^{*}p \leq 0.05$. (B) miR-148a expression in *Tbx21*$^{-/-}$ Th1 cells and wt control (*Tbx21*$^{+/+}$) 48 h after activation, assessed by qRT-PCR, normalized to snU6 and presented relative to values obtained in naive *Tbx21*$^{+/+}$ or *Tbx21*$^{-/-}$ Th cells. Data are shown as mean ± SEM, $n = 11$ (*Tbx21*$^{+/+}$) and $n = 12$ (*Tbx21*$^{-/-}$), pooled from three independent experiments. (C) Overexpression of T-bet and a T-bet mutant (T-bet Y182S) in activated Th1 cells, assessed by qRT-PCR, analyzed on day 5 postactivation. miR-148a was normalized to snU6 and presented relative to values obtained with an empty retroviral control vector (RV). Data are shown as mean ± SEM, $n = 8$ (RV), $n = 7$ (T-bet), and $n = 6$ (T-bet Y182S), pooled from three independent experiments. (D) *Twist1* expressionin once and repeatedly activated Th1 cells, assessed by qRT-PCR, normalized to HPRT, and presented relative to values obtained for naive Th cells. Each data point represents an individual experiment, performed with $n = 1$ (Wilcoxon-test for paired data, $^{*}p \leq 0.05$). (E) *Bim* expression in ex vivo isolated *Twist1*$^{fl/fl}$ CD4-Cre$^{+/-}$ cells and *Twist1*$^{wt/wt}$ CD4-Cre$^{+/-}$ control, assessed by qRT-PCR, normalized to HPRT and presented relative to values obtained for *Twist1*$^{wt/wt}$ CD4-Cre$^{+/-}$. Data are shown as mean ± SEM, $n = 1$, each pooled from two independent experiments. (F) miR-148a expression in repeatedly activated *Twist1*$^{fl/fl}$ CD4-Cre$^{+/-}$ Th1 cells and *Twist1*$^{wt/wt}$ CD4-Cre$^{+/-}$ control, assessed by qRT-PCR, normalized to snU6 and presented relative to values obtained for *Twist1*$^{wt/wt}$ CD4-Cre$^{+/-}$. Data are shown as mean ± SEM, $n = 1$, each pooled from three independent experiments. (G) Twist1 binding to miR-148a locus determined by chromatin immunoprecipitation (ChIP), normalized to total DNA and presented relative to samples obtained from *Twist1*$^{fl/fl}$ CD4-Cre$^{+/-}$ Th1cells. Data are shown as mean ± SEM, $n = 1$, each pooled from four independent experiments. (B, C, and E–G) Mann–Whitney test for unpaired data, $^{*}p \leq 0.05$, $^{**}p \leq 0.005$, $^{***}p \leq 0.001$.

T-bet mutant Y182S [26] significantly reduced miR-148a expression (Fig. 5C). In repeatedly activated Th1 cells we observed a 12-fold induction of expression of the transcription factor *Twist1* (Fig. 5D) [9]. *Twist1*-deficient Th cells isolated ex vivo showed a significant reduction in miR-148a expression (data not shown), while *Bim* levels were significantly increased by 60%, as compared to *Twist1*-sufficient cells (Fig. 5E). In vitro, repeatedly activated *Twist1*-deficient Th1 cells showed a threefold decreased miR-148a expression compared to *Twist1*-sufficient cells (Fig. 5F). Chromatin immunoprecipitation (ChIP) of Twist1 revealed a functional Twist1 bs 2.1 kb upstream of the miR-148a gene (Fig. 5G and Supporting Information Fig. 6). We used the IFN-γ promoter (IFN-γ -0.4) and CNS-34 (IFN-γ -34)[27], both containing E-box binding sites, as positive controls. These results imply an indirect role of T-bet and a direct involvement of Twist1 in regulating miR-148a expression in Th1 cells.

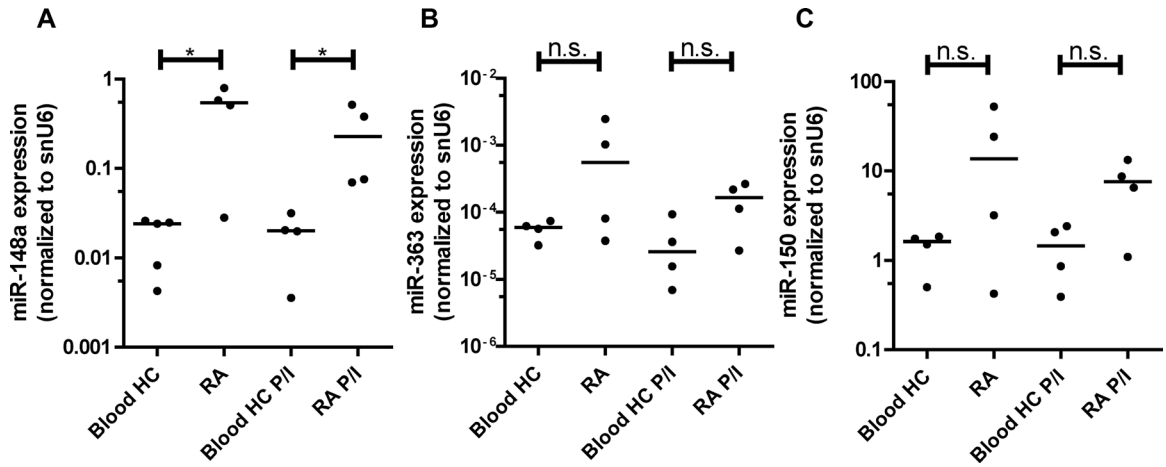


Figure 6. miR-148a is expressed in patients suffering from rheumatoid arthritis. (A) miR-148a, (B) miR-363, and (C) miR-150 expression, assessed by qRT-PCR, in $CD3^+CD4^+CD14^-CD45RO^+$ T cells isolated from synovial fluids of patients suffering from rheumatoid arthritis (RA) or blood from healthy control (HC) donors either ex vivo ($n = 4$ RA / $n = 5$ Blood HC) or after 3 h of mitogenic restimulation with PMA/Ionomycin (P/I) ($n = 4$ RA P/I / $n = 4$ Blood HC P/I), normalized to snU6. Data are representative of two independent experiments; each data point represents an individual donor, horizontal bar; median. (Mann–Whitney test for unpaired data, $^*p \leq 0.05$).

miR-148a is expressed in T cells from rheumatoid arthritis patients

$CD3^+CD4^+CD14^-CD45RO^+$ T cells, isolated from inflamed tissue of patients suffering from rheumatic arthritis (RA) exhibit significantly higher levels of miR-148a, both directly ex vivo as well as after 3 h of mitogenic restimulation with PMA/Ionomycin, compared to cells isolated from peripheral blood of healthy donors (Fig. 6A). To demonstrate the selectivity of this upregulation, we also quantified miR-363 and miR-150 expression in these cells [28]. We did not detect significant differential expression between cells from peripheral blood and cells from synovial fluid, isolated ex vivo (Fig. 6B and C). Memory/effector cells isolated from the synovial fluid of arthritic patients are enriched for Th1 cells, as assessed by chemokine receptor expression, including CXCR3, CCR5, CCR4, and CCR6 [29] (data not shown). A positive correlation of miR-148a expression and *Twist1* expression in these cells was observed (Supporting Information Fig. 6B), further supporting the role of Twist1 as a key inducer of miR-148a expression.

Discussion

We have previously shown that the transcription factor Twist1 (i) is upregulated in repeatedly activated Th1 cells, (ii) is highly upregulated in restimulated $CD3^+CD4^+$ cells isolated from the inflamed tissue of patients with chronic inflammation of the joint, and (iii) limits immunopathology in a murine model of arthritis [9]. Here we show that Twist1, together with T-bet, also controls the persistence of repeatedly activated Th1 cells, by upregulating expression of miR-148a, which in turn targets the proapoptotic gene *Bim*.

We have previously demonstrated that Twist1 is only expressed in Th1 cells, as its induction was directly dependent on IL-12/STAT4 signaling in combination with nuclear factor of activated T cells (NFAT) and nuclear factor κB (NF-κB) [9]. In Th1 cells, the expression of Twist1 gradually increased upon repeated rounds of restimulation, which is in line with the induction of miR-148a in repeatedly activated Th1 cells. In such cells, Twist1 binds to a functional E-box motif 2.1 kb upstream of the miR-148a gene and presumably directly regulates miR-148a expression. We also observed a contribution of T-bet on the expression of miR-148a by analyzing *Tbx21*-deficient Th cells as well as by ectopic overexpression of T-bet and a dominant-negative T-bet mutant (T-bet Y182S) in vitro. Since we could not identify potential T-bet binding sites in the regulatory elements of the miR-148a locus, we suggest that T-bet enhances expression of miR-148a indirectly. This is in line with the results of Nakayamada and colleagues, who have shown that T-bet does not bind within a 200 kb region upstream of the miR-148a locus [25]. T-bet is not required for the induction of Twist1 [9], but it may induce expression of an as yet unknown transcription factor required for miR-148a transcription.

Expression of miR-148a is not only induced in Th cells activated and polarized in vitro to develop into Th1 cells, but also in murine LCMV-specific Th1 cells generated in vivo, upon infection of mice with LCMV. In humans, we had described earlier, that *TWIST1* expression is significantly upregulated in Th cells isolated from inflamed tissues of patients with chronic inflammatory diseases, as compared to Th cells from blood or healthy colon [9]. Here we show that Th cells isolated from synovial fluid of patients with rheumatoid arthritis have upregulated expression of miR-148a. Interestingly, upregulation of miR-148a correlates with the expression levels of *TWIST1*. miR-148a-mediated suppression of Bim, as discussed below, does promote the persistence and viability of the cells and may contribute to the resistance of the cells toward currently available therapies. Thus, Twist1 has a dual role as (i) an attenuator of Th1 effector function, to minimizing immunopathology, and (ii) a promotor of persistence of these

Th1 cells in situations of repeated restimulation. These functions identify Twist1 as a master switch of chronicity in inflammation.

The proapoptotic protein Bim regulates the survival of memory Th cells by antagonizing the antiapoptotic protein Bcl2 [15]. The molecular mechanisms controlling the critical balance between Bcl2 and Bim in T cells are poorly understood. In reactivated Th1 cells as compared to Th17 cells, Bcl2 is downregulated [30]. This suppression of Bcl2 is probably mediated by T-bet. T-bet suppresses IL-2 expression [17], and IL-2 is required for the upregulation of Bcl2 [31]. Thus, in order to survive and maintain an antiapoptotic Bim/Bcl2 ratio, Th1 cells have to suppress expression of Bim, too. As we show here, in repeatedly activated Th1 cells, an efficient mechanism to suppress Bim expression is its posttranscriptional downregulation by miR-148a, which is induced by T-bet and Twist1. T-bet and Twist1 are key transcription factors of Th1 cells. T cells of other differentiation lineages seem to have developed different mechanisms and depend less on activation induced cell death. Repeatedly activated Th17 cells upregulate Bcl2 expression [30, 32] and downregulate the Fas-L [33–35] when compared to reactivated Th1 cells. Likewise, Th2 cells maintain or upregulate the expression of Bcl2 via IL-4 signaling [36, 37] and reduce Fas and FAS-L expression [38–40]. Neither of the T cell lineages upregulates miR-148a expression. This is in line with our observation, that antagomir-148a treatment does not influence the survival of repeatedly activated Th2 and Th17 cells.

We here demonstrate that Bim is the main target of miR-148a regarding the control of survival of Th1 cells. Complementing the antagomir-mediated inhibition of miR-148a with siRNA-mediated suppression of Bim largely restores viability of the Th1 cells. The physiological level of miR-148a in repeatedly activated Th1 cells is apparently phylogenetically optimized for the suppression of Bim with regard to its role in maintaining viability. SiRNA-mediated reduction of Bim expression in repeatedly activated Th1 cells with endogenous levels of miR-148a expression did not further increase the number of viable cells. Our results are supported by the notion that miR-148a supports the survival of glioblastoma cells by targeting *BIM* [41].

Theoretically, other miRNAs could possibly target Bim in repeatedly activated Th1 cells, for example, miRNAs of the miR-17-92 cluster [42]. However, among the 13 miRNAs selectively upregulated by repeatedly activated Th1 cells, as compared to Th2 and Th17 cells, none, except miR-148a, has candidate seed sequences in the 3′-UTR of Bim. miRNAs of the miR-17-92 cluster are not upregulated (data not shown). Therefore, we conclude that posttranscriptional regulation of Bim in repeatedly activated Th1 cells is mediated by miR-148a.

For miR-148a other potential targets affecting viability have been described for other cell types, namely, Pten and Bcl2 for hepatocytes and colorectal cancer cells, respectively [24, 43]. However, both *Pten* and *Bcl2* are not differentially expressed between once and repeatedly activated Th1 cells. Expression of Bcl2 protein is increased by 10% in repeatedly activated Th1 cells upon miR-148a inhibition by antagomirs. Since *Bcl2* mRNA levels do not change detectably, the difference in protein expression, tiny as it is, could be due to either inhibition of translation of *Bcl2* mRNA by miR-148a at physiological levels, or to enhanced stabilization of Bcl2 protein by the elevated levels of Bim protein [44] in antagomir-148a treated Th1 cells. With respect to Pten, a negative regulator of cell cycle progression and indirect inducer of Bim expression [45, 46], we did not observe any effect on its endogenous expression levels when we blocked miR-148a in repeatedly activated Th1 cells. Although miR-148a overexpression in once activated Th1 cells regulated expression of a reporter gene with the Pten 3′-UTR, in dependency of the presence of the miR-148a target sequence, endogenous Pten levels were not significantly affected. Thus, the lack of regulation of Pten by miR-148a in repeatedly activated Th1 cells either reflects a context-dependent activity of miR-148a, or the quantitative difference of endogenous versus ectopic miR-148a expression levels. In any case, physiological expression of miR-148a in repeatedly activated Th1 cells does not regulate Pten expression.

Repeatedly activated Th1 cells express candidate target genes of miR-148a, which affect biological functions other than survival (Supporting Information Table 1). These genes influence functions such as migration (e.g. S1PR1 and RAB11B), differentiation (e.g. Bach2) and miRNA maturation (Dicer1). Not all of them may be true targets of miR-148a in the context of repeatedly activated Th1 cells. In the accompanying paper by Porstner et al., the authors identified *Mitf* and *Bach2* as targets of miR-148a in B lymphocytes. miR-148a promotes the differentiation of activated B cells into plasma cells, by repressing *Bach2* and *Mitf*. The transcription factor *Mitf* is neither expressed in once nor in repeatedly activated Th1 cells. Bach2 is a transcriptional repressor involved in regulation of effector functions in Th cells [47]. Bach2 expression is inversely correlated to miR-148a expression in Th1 cells. However, inhibition of miR-148a in repeatedly activated Th1 cells did not increase expression of *Bach2*, suggesting that Bach2 in these cells is not a physiological target. The present data confirm that it is essential to analyze target gene regulation by miRNAs in under physiological conditions. While inhibition of miR-148a in these cells sufficed to regulate Bim, no effect on the expression of Pten, Bach2, and Bcl2 was observed, all of which are described targets of miR-148a in other cells [24, 43] (accompanying paper by Porstner et al.). Beyond its significant effect on viability, by regulation of Bim, it remains to be shown, to what extend miR-148a is regulating function and fate of Th1 cells in chronic or recurrent immune reactions.

Materials and methods

Mice

OTII, C57BL/6, BALB/c, C57BL/6^{SMARTA}, Twist$^{fl/fl}$ CD4-Cre$^{+/-}$, Twist$^{WT/WT}$ CD4-Cre$^{+/-}$, and *Tbx21*$^{-/-}$ mice were housed and bred under specific pathogen-free conditions. Mice were handled in accordance with good animal practice as defined by German animal welfare bodies and sacrificed by cervical dislocation. All experiments were approved by the federal state institution

"Landesamt für Gesundheit und Soziales" (G0325/12) in Berlin, Germany.

Cell culture

Naïve or $CD4^+$ T cells were isolated from spleens of 6–10 weeks old mice and cultured under Th1, Th2, and Th17 inducing conditions as described in [48]. In brief, 3×10^6 Th cells/mL were cultured and stimulated in the presence of 0.5 mM cognate peptide OVA323–339 for OT-II cells or plate-bound αCD3/αCD28 (3 μg/mL, EBioscience) for C57BL/6, BALB/c, $Twist^{fl/fl}$ $CD4\text{-}Cre^{+/-}$, and $Tbx21^{-/-}$ cells. If T cells were cultured for more than 5 days irradiated (30 Gy) CD90 depleted splenocytes from C57BL/6 or BALB/c mice were added as antigen-presenting cells to the culture. Th1 differentiation was achieved by addition of recombinant IL-12 (5 ng/mL; R&D Systems, Minneapolis, MN) and anti-IL-4 (11B11) antibody. Th2 differentiation was achieved by addition of IL-4 (100 ng/mL, culture supernatant of HEK293 T cells transfected with murine IL-4 cDNA), anti-IL-12 (C17.8), and anti-IFN-γ (AN18.17.24) antibodies. Th17 differentiation was achieved by addition of TGF-β1 (1 ng/mL), IL-6, IL-23 (20 ng/mL) (all from R&D Systems), anti-IL-4 and anti-IFN-γ. Cells were restimulated every 6 days for three times to induce a chronically activated phenotype. Recombinant IFN-γ (rIFN-γ) was added at 10 ng/mL in *Tbx21* knockout experiments.

Patient material

Synovial fluids were taken from patients suffering from rheumatoid arthritis, psoriatic arthritis (PsA) or juvenile idiopathic arthritis (JA) by puncture of joints. Fluids were mixed and washed with PBS/EDTA. Cell suspension was depleted of $CD15^+$ cells using MACS and sorted for $CD3^+$ $CD4^+$ $CD14^-$ $CD45RO^+$ cells using FACS. Blood was taken from healthy donors as control. Cells were isolated by density gradient separation using LSM 1077 Lymphocyte separation medium and sorted for $CD3^+$ $CD4^+$ $CD14^-$ $CD45RO^+$ cells using FACS. Half the cells were restimulated with PMA/Ionomycin for 3 h. Samples were lysed in TRIzol Reagent (Invitrogen) for RNA extraction. All human studies were approved by the Charité ethical committee and the informed consent of all participating subjects was obtained.

LCMV model

$Thy1.1^+$ spleenocytes from $C57BL/6^{SMARTA}$ were naive sorted by depletion of CD11c, CD11b, CD19, Gr1, CD8, NK1.1, CD25 positive cells. A total of 5×10^6 naive $CD4^+$ SM TCRtg T cells was transferred intravenously into C57BL/6 host mice and 2 days after transfer, mice were infected with 200 plaque-forming units of LCMV strain WE. Mice were sacrificed on day 5 and day 7, sorted for $Thy1.1^+$, $CD44^{high}$, $CD62L^{low}$ cells and analyzed for miR-148a expression. T-bet expression was determined by flow cytometry in blood of animals prior to sacrifice. All animal experiments were in accordance with institutional, state and federal guidelines (Landesamt Für Gesundheit und Soziales, Berlin, Germany, accreditation number G0325/12).

Retroviral transfection

Viral supernatants were produced using 293 HEK cells transfected with pECO, pCGP, and respective transfer plasmid [9]. $CD4^+$ T cells were isolated and activated as described above. Thirty six hours postactivation RPMI medium was removed and virus-containing medium supplemented with HEPES-buffer (20 mM) and polybrene (8 μg/mL) was added to T cells. Cells were centrifuged for 1.5 h at 32°C, 1800 rpm, supernatant was removed and Th1 medium was readded.

Plasmids

A miR-148a overexpression vector was generated by amplifying a primary form of miR-148a from murine spleen cDNA using the following primers: pri148a forward 5′-GTTAACTGTGACATTGCCACCAGA-3′ and pri148a reverse 5′-CTCGAGAAAAAAAACGACGTGGCCAACA-3′; and cloned under the control of U6 promoter into pQCXIX (BD Biosciences Clontech) using *Hpa*I and *Xho*I restriction enzymes. pQCXIX has a GFP marker for positive selection. As a control, a scrambled miRNA overexpression vector was constructed as described before [22]. Transfection of T cells was performed as described above. Three days posttransfection cells were harvested and sorted for GFP expression. miRNA expression levels were assessed by quantitative PCR. For cotransfection with reporter vectors, cells were analyzed by flow cytometry. For generation of *Bim* and *Pten* reporter vectors, parts of the respective 3′-UTRs were amplified from cDNA using the following primers: BIM forward 5′-CGCGGATCCCTCAAGTTCCCAGCAAAGTA-3′, BIM reverse 5′-CCCAAGCTTCACAGGTACAGTGGCAATTA-3′, PTEN forward 5′-CGCGGATCCGCTGAAAGTGGCTGACTAAA-3′, and PTEN reverse 5′-CCCAAGCTTCACCCACACAATGACAAGA-3′; and cloned downstream of the human CD4 gene within the pMSCV vector (BD Biosciences Clontech). Mutation of miR-148a binding sites (bs) in 3′-UTRs of *Bim* and *Pten* reporter vectors were induced using the site specific mutagenesis kit (Stratagene) and the following primer:

BIM1 mut forward 52-GGTATCCTTTAGTGAACAGCGGTCGTC TCTGTATAGTCCCCATCAC-32, BIM1 mut reverse 52-GTGATGGG GACTATACAGAGACGACCGCTGTTCACTAAAGGATACC-3′, BIM2 mut forward 52-CTGGCTTCCTTTACGTTTTGCGGCCATGAATTTT GACAGGGTAATTGC-32, BIM2 mut reverse 52-GCAATTACCCT GTCAAAATTCATGGCCGCAAAACGTAAAGGAAGCCAG-322, PTEN2 mut forward 52-GCAGTGGCTCTGTGTGTAAATGCTA GCCACGCAGGATACACACAAATATG-32, PTEN2 mut forward 52-CATATTTGTGTATCCTGCGTGGCTAGCATTTACACACAGAGCCAC TGC-32. Transfection of T cells was performed as described above.

T cells were stained with αhCD4-Cy5 (Clone TT1, purified in-house) and expression of human CD4 reporter gene was assessed by flow cytometry as described previously [22].

Inhibition of miR-148a

Inhibition of miR-148a was achieved using specific cholesterol-coupled antagomir oligonucleotides (custom synthesized by Dharmacon) [23]. Repeatedly activated Th1 cells were resuspended in serum-free medium (ACCELL, Dharmacon) supplemented with antagomir-148a or scrambled control (1 μM). After 1.5 h incubation at 37°C and 5% CO_2 T cells were reactivated and cultured in Th1 polarizing medium (added in fourfold excess to T cells in ACCELL medium). Knock down efficiency was assessed by quantitative PCR.

Inhibition of Bim and Twist1

Specific ACCELL siRNAs targeting *Bim* (Dharmacon) were used to decrease functional *Bim* mRNA expression. Repeatedly activated Th1 cells were treated with 1 μM antagomir and 1 μM siRNA in serum-free ACCELL medium. After 1.5 h incubation at 37°C and 5% CO_2 T cells were reactivated and cultured in Th1 polarizing medium (added 1:1 to a final concentration of 0.5 μM antagomir and siRNA). Knockdown efficiency was assessed by qRT-PCR and phenotypical effects were detected by flow cytometry. *Twist1* knockdown was achieved by retroviral expression of shRNA as described before [9].

Apoptosis assay

T cells were stained with αmCD4-Cy5 (GK1.5; purified in-house). Apoptosis was examined using an AnnexinV-FITC/Propidiumiodide Assay (Roche) and analyzed by flow cytometry.

Total numbers of viable T cells were determined by flow cytometry (MACSQuant Analyzer, Miltenyi Biotec).

Chromatin immunoprecipitation

ChIP was performed as described previously [48] with a polyclonal Twist1 antibody (4 μg/mL; sc-6070; Santa Cruz Biotechnology). Immunoprecipitated DNA was measured by quantitative PCR. Putative binding sites in conserved regions of the miR-148a locus (chromosome 6; 51269812-51269910 (–)) were identified with the web server of the comparative tool rVista based on the professional V10.2 library of the TRANSFAC database. The following primers were used: miR-148 -0.1kb forward 5′- CCTCTGGAAGTTTCGTCCTGC-3′, miR-148 -0.1kb reverse 5′-TATTCTTCTTTGCCTTCACTGGG-3′, miR-148 -1.4kb forward 5′-ATTTGGGTTTGGAGACGACC-3′, miR-148 -1.4kb reverse 5′-AATAGCAAGAGCAGCCGTGAC-3′, miR-148 -1.7kb forward 5′-AGCAGAGTGAGAAATGGAAACCTT-3′, miR-148 -1.7kb reverse 5′-TCTCAGTTCTTGTAACACTCAGCCC-3′, miR-148 -2.1kb forward 5′-AACTCAAGGTGCTCAGAATTGTCC-3′, miR-148 -2.1kb reverse 5′-CCTTTCTTCTACAAAGCACGCCT-3′. Regulatory regions of the IFN-γ promoter described by [27] served as positive controls.

RNA extraction and quantitative PCR

Total RNA was isolated with RNeasy kit (Qiagen) or Direct-zol RNA kit (Zymo Research). Mature miR-148a and U6 small nuclear RNA (snRNA) were detected by quantitative PCR with Taqman MicroRNA Reverse Transcription kit in combination with TaqMan MicroRNA Assays (Applied Biosystems) according to manufacturer's recommendations. For normalization the expression values were compared to values of snU6 by the change-in-threshold method ($2^{-\Delta CT}$).

Reverse transcription of mRNA was performed using the Reverse Transcription kit (Applied Biosystems) and cDNA was quantified by SYBR Green based real-time PCR (Roche) using the following primer pairs: hypoxanthine guanine phosphoribosyltransferase (HPRT) forward 5′-TCCTCCTCAGAC CGCTTTT-3′, HPRT reverse 5′-CATAACCTGGTTCATCATCGC-3′, BIM forward 5′-CCCGGAGATACGGATTGCA-3′, BIM reverse 5′-AA CACCCTCCTTGTGTAAGTTTCGT-3′, BCL2 forward 5′-TGAACCG GCATCTGCACA -3′, BCL2 reverse 5′-CAGAGGTCGCATGCTGGG-3′, PTEN forward 5′-GCGGAACTTGCAATCCTCAGT-3′, PTEN reverse 5′- AGGCAATGGCTGAGGGAACT-3′, TBET forward 5′-TCCTGCAGTCTCTCCACAAGT-3′, TBET reverse 5′-CAGCTG AGTGATCTCTGCGT-3′, TWIST1 forward 5′-CGCACGCAGT CGCTGAACG-3′, TWIST1 reverse 5′-GACGCGGACATGGACCAGG -3′. For normalization, the expression values were compared to values of HPRT by the change-in-threshold method ($2^{-\Delta CT}$).

Immunoblot analysis

Immunoblot analysis was performed as described previously [49]. Membranes were probed with primary antibodies to total Pten (D4.3, Cell Signaling), Bim (3C5, Enzo Life Sciences) and Bcl2 (50E3, Cell Signaling), and polyclonal antibody to actin (sc-1616; Santa Cruz) for normalization. Immunoreactive bands were detected by chemiluminescence using ECL reagent (GE Healthcare) and quantified by densitometry (Fuji LAS-4000 software).

Intracellular protein/cytokine staining

Intracellular cytokine staining was performed after every round of (re)stimulation. Cells were stimulated with PMA/Ionomycin for 3 h and fixed in 2% paraformaldehyde. Cytokine specific antibodies were administered in 0.5% Saponin in PBS, incubated for 20 min at 4°C and analyzed by flow cytometry. Intracellular protein staining was performed using primary antibodies to Bim (14A8, Merck Chemicals) and Bcl2 (3F11, BD Biosciences). Cells were

fixed and stained using the Foxp3 staining buffer set (eBioscience) according to manufacturer's recommendation with extended fixation overnight. Samples were stained for 30 min at 4°C and analyzed by flow cytometry.

Isolation of IFN-γ-secreting cells

Separation of IFN-γ-producing and nonproducing Th1 cells was performed in principle as described previously [50–52]. Once activated Th1 cells were restimulated with PMA/Ionomycin for 3 h. IFN-γ-catch and -detection reagents (aIFN-γ-allophycocyanin) were obtained from Miltenyi Biotec and used according to the manufacturer's guidelines.

CFSE dilution assay

To test the proliferative capacity of antagomir-148a or antagomir-scr treated Th1 rep. cells, we performed a CFSE dilution assay according to a published protocol [53].

Statistics

All statistical analyses used the Mann–Whitney test for unpaired data, unless stated otherwise. A p-value of equal or less than 0.05 was considered significant.

Acknowledgements: This work was supported by the Deutsche Forschungsgemeinschaft (GRK1121; BSRT (DFG-GSC203), SFB 618, SFB 650, SFB 633, and SFB TR52), the International Max Planck Research School for Infectious Diseases and Immunology, the IMI JU funded project BTCure, no 115241, the FORSYS (Forschungseinheiten zur Systembiologie)and the "e:Bio – Innovationswettbewerb Systembiologie" program of the Federal Ministry of Education. J.W. and H.-M.J. were supported in part by the Interdisciplinary Center for Clinical Research (IZKF) Erlangen (project D7) and research grants GRK1660, FOR832 (JA968/4) and TRR130 from the Deutsche Forschungsgemeinschaft (DFG). We would like to thank Jenny Kirsch and Toralf Kaiser for technical support. This work has been supported by the ERC Advanced Grant (ERC-2010-AdG_20100317 Grant 268987) to A.R.

Conflict of interest: The authors declare no commercial or financial conflict of interest.

References

1 **Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. and Coffman, R. L.**, Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* 1986. **136**: 2348–2357.

2 **Schulz, E. G., Mariani, L., Radbruch, A. and Hofer, T.**, Sequential polarization and imprinting of type 1 T helper lymphocytes by interferon-gamma and interleukin-12. *Immunity* 2009. **30**: 673–683.

3 **Neurath, M. F., Weigmann, B., Finotto, S., Glickman, J., Nieuwenhuis, E., Iijima, H., Mizoguchi, A. et al.**, The transcription factor T-bet regulates mucosal T cell activation in experimental colitis and Crohn's disease. *J. Exp. Med.* 2002. **195**: 1129–1143.

4 **Janke, M., Peine, M., Nass, A., Morawietz, L., Hamann, A. and Scheffold, A.**, In-vitro-induced Th17 cells fail to induce inflammation in vivo and show an impaired migration into inflamed sites. *Eur. J. Immunol.* 2010. **40**: 1089–1098.

5 **Bettelli, E., Sullivan, B., Szabo, S. J., Sobel, R. A., Glimcher, L. H. and Kuchroo, V. K.**, Loss of T-bet, but not STAT1, prevents the development of experimental autoimmune encephalomyelitis. *J. Exp. Med.* 2004. **200**: 79–87.

6 **Fuss, I. J., Neurath, M., Boirivant, M., Klein, J. S., de la Motte, C., Strong, S. A., Fiocchi, C. et al.**, Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. *J. Immunol.* 1996. **157**: 1261–1270.

7 **Morita, Y., Yamamura, M., Kawashima, M., Harada, S., Tsuji, K., Shibuya, K., Maruyama, K. et al.**, Flow cytometric single-cell analysis of cytokine production by CD4+ T cells in synovial tissue and peripheral blood from patients with rheumatoid arthritis. *Arthritis Rheum.* 1998. **41**: 1669–1676.

8 **Yamada, H., Nakashima, Y., Okazaki, K., Mawatari, T., Fukushi, J. I., Kaibara, N., Hori, A. et al.**, Th1 but not Th17 cells predominate in the joints of patients with rheumatoid arthritis. *Ann. Rheum. Dis.* 2008. **67**: 1299–1304.

9 **Niesner, U., Albrecht, I., Janke, M., Doebis, C., Loddenkemper, C., Lexberg, M. H., Eulenburg, K. et al.**, Autoregulation of Th1-mediated inflammation by twist1. *J. Exp. Med.* 2008. **205**: 1889–1901.

10 **Snow, A. L., Pandiyan, P., Zheng, L., Krummey, S. M. and Lenardo, M. J.**, The power and the promise of restimulation-induced cell death in human immune diseases. *Immunol. Rev.* 2010. **236**: 68–82.

11 **Kurtulus, S., Tripathi, P., Opferman, J. T. and Hildeman, D. A.**, Contracting the 'mus cells'–does down-sizing suit us for diving into the memory pool? *Immunol. Rev.* 2010. **236**: 54–67.

12 **Shenoy, A. R., Kirschnek, S. and Hacker, G.**, IL-15 regulates Bcl-2 family members Bim and Mcl-1 through JAK/STAT and PI3K/AKT pathways in T cells. *Eur. J. Immunol.* 2014. **44**: 2500–2507.

13 **Pellegrini, M., Belz, G., Bouillet, P. and Strasser, A.**, Shutdown of an acute T cell immune response to viral infection is mediated by the proapoptotic Bcl-2 homology 3-only protein Bim. *Proc. Natl. Acad. Sci. U S A* 2003. **100**: 14175–14180.

14 **Adams, J. M. and Cory, S.**, Bcl-2-regulated apoptosis: mechanism and therapeutic potential. *Curr. Opin. Immunol.* 2007. **19**: 488–496.

15 **Wojciechowski, S., Tripathi, P., Bourdeau, T., Acero, L., Grimes, H. L., Katz, J. D., Finkelman, F. D. et al.**, Bim/Bcl-2 balance is critical for maintaining naive and memory T cell homeostasis. *J. Exp. Med.* 2007. **204**: 1665–1675.

16 **Lai, Y. G., Hou, M. S., Lo, A., Huang, S. T., Huang, Y. W., Yang-Yen, H. F. and Liao, N. S.**, IL-15 modulates the balance between Bcl-2 and Bim via a Jak3/1-PI3K-Akt-ERK pathway to promote CD8alphaalpha+ intestinal intraepithelial lymphocyte survival. *Eur. J. Immunol.* 2013. **43**: 2305–2316.

17 **Szabo, S. J., Kim, S. T., Costa, G. L., Zhang, X., Fathman, C. G. and Glimcher, L. H.**, A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 2000. **100**: 655–669.

18 **Rebhahn, J. A., Deng, N., Sharma, G., Livingstone, A. M., Huang, S. and Mosmann, T. R.**, An animated landscape representation of CD4(+) T-cell differentiation, variability, and plasticity: Insights into the behavior of populations versus cells. *Eur. J. Immunol.* 2014. **44**: 2216–2229.

19 **Kozomara, A. and Griffiths-Jones, S.**, miRBase: integrating microRNA annotation and deep-sequencing data. *Nucleic Acids Res.* 2011. **39**: D152–157.

20 **Krek, A., Grun, D., Poy, M. N., Wolf, R., Rosenberg, L., Epstein, E. J., MacMenamin, P. et al.**, Combinatorial microRNA target predictions. *Nat. Genet.* 2005. **37**: 495–500.

21 **Lewis, B. P., Shih, I. H., Jones-Rhoades, M. W., Bartel, D. P. and Burge, C. B.**, Prediction of mammalian microRNA targets. *Cell* 2003. **115**: 787–798.

22 **Stittrich, A. B., Haftmann, C., Sgouroudis, E., Kuhl, A. A., Hegazy, A. N., Panse, I., Riedel, R. et al.**, The microRNA miR-182 is induced by IL-2 and promotes clonal expansion of activated helper T lymphocytes. *Nat. Immunol.* 2010. **11**: 1057–1062.

23 **Krutzfeldt, J., Rajewsky, N., Braich, R., Rajeev, K. G., Tuschl, T., Manoharan, M. and Stoffel, M.**, Silencing of microRNAs in vivo with 'antagomirs'. *Nature* 2005. **438**: 685–689.

24 **Yuan, K., Lian, Z., Sun, B., Clayton, M. M., Ng, I. O. and Feitelson, M. A.**, Role of miR-148a in hepatitis B associated hepatocellular carcinoma. *PLoS One* 2012. **7**: e35331.

25 **Nakayamada, S., Kanno, Y., Takahashi, H., Jankovic, D., Lu, K. T., Johnson, T. A., Sun, H. W. et al.**, Early Th1 cell differentiation is marked by a Tfh cell-like transition. *Immunity* 2011. **35**: 919–931.

26 **Miller, S. A., Huang, A. C., Miazgowicz, M. M., Brassil, M. M. and Weinmann, A. S.**, Coordinated but physically separable interaction with H3K27-demethylase and H3K4-methyltransferase activities are required for T-box protein-mediated activation of developmental gene expression. *Genes Dev.* 2008. **22**: 2980–2993.

27 **Chang, S. and Aune, T. M.**, Dynamic changes in histone-methylation 'marks' across the locus encoding interferon-gamma during the differentiation of T helper type 2 cells. *Nat. Immunol.* 2007. **8**: 723–731.

28 **Li, J., Wan, Y., Guo, Q., Zou, L., Zhang, J., Fang, Y., Zhang, J. et al.**, Altered microRNA expression profile with miR-146a upregulation in CD4+ T cells from patients with rheumatoid arthritis. *Arthritis Res. Ther.* 2010. **12**: R81.

29 **Cosmi, L., Cimaz, R., Maggi, L., Santarlasci, V., Capone, M., Borriello, F., Frosali, F. et al.**, Evidence of the transient nature of the Th17 phenotype of CD4+CD161+ T cells in the synovial fluid of patients with juvenile idiopathic arthritis. *Arthritis Rheum.* 2011. **63**: 2504–2515.

30 **Muranski, P., Borman, Z. A., Kerkar, S. P., Klebanoff, C. A., Ji, Y., Sanchez-Perez, L., Sukumar, M. et al.**, Th17 cells are long lived and retain a stem cell-like molecular signature. *Immunity* 2011. **35**: 972–985.

31 **Chetoui, N., Boisvert, M., Gendron, S. and Aoudjit, F.**, Interleukin-7 promotes the survival of human CD4+ effector/memory T cells by upregulating Bcl-2 proteins and activating the JAK/STAT signalling pathway. *Immunology* 2010. **130**: 418–426.

32 **Kitajima, M., Lee, H. C., Nakayama, T. and Ziegler, S. F.**, TSLP enhances the function of helper type 2 cells. *Eur. J .Immunol.* 2011. **41**: 1862–1871.

33 **Shi, G., Ramaswamy, M., Vistica, B. P., Cox, C. A., Tan, C., Wawrousek, E. F., Siegel, R. M. et al.**, Unlike Th1, Th17 cells mediate sustained autoimmune inflammation and are highly resistant to restimulation-induced cell death. *J. Immunol.* 2009. **183**: 7547–7556.

34 **Annunziato, F., Cosmi, L., Liotta, F., Maggi, E. and Romagnani, S.**, Type 17 T helper cells-origins, features and possible roles in rheumatic disease. *Nat. Rev. Rheumatol.* 2009. **5**: 325–331.

35 **Tan, C., Ramaswamy, M., Shi, G., Vistica, B. P., Siegel, R. M. and Gery, I.**, Inflammation-inducing Th1 and Th17 cells differ in their expression patterns of apoptosis-related molecules. *Cell. Immunol.* 2011. **271**: 210–213.

36 **Dunkle, A., Dzhagalov, I. and He, Y. W.**, Cytokine-dependent and cytokine-independent roles for Mcl-1: genetic evidence for multiple mechanisms by which Mcl-1 promotes survival in primary T lymphocytes. *Cell. Death Dis.* 2011. **2**: e214.

37 **Rautajoki, K. J., Marttila, E. M., Nyman, T. A. and Lahesmaa, R.**, Interleukin-4 inhibits caspase-3 by regulating several proteins in the Fas pathway during initial stages of human T helper 2 cell differentiation. *Mol. Cell. Proteomics* 2007. **6**: 238–251.

38 **Pandiyan, P., Gartner, D., Soezeri, O., Radbruch, A., Schulze-Osthoff, K. and Brunner-Weinzierl, M. C.**, CD152 (CTLA-4) determines the unequal resistance of Th1 and Th2 cells against activation-induced cell death by a mechanism requiring PI3 kinase function. *J. Exp. Med.* 2004. **199**: 831–842.

39 **Zhang, X., Brunner, T., Carter, L., Dutton, R. W., Rogers, P., Bradley, L., Sato, T. et al.**, Unequal death in T helper cell (Th)1 and Th2 effectors: Th1, but not Th2, effectors undergo rapid Fas/FasL-mediated apoptosis. *J. Exp. Med.* 1997. **185**: 1837–1849.

40 **Varadhachary, A. S., Perdow, S. N., Hu, C., Ramanarayanan, M. and Salgame, P.**, Differential ability of T cell subsets to undergo activation-induced cell death. *Proc. Natl. Acad. Sci. U S A* 1997. **94**: 5778–5783.

41 **Homann, D., Teyton, L. and Oldstone, M. B.**, Differential regulation of antiviral T-cell immunity results in stable CD8+ but declining CD4+ T-cell memory. *Nat. Med.* 2001. **7**: 913–919.

42 **Xiao, C., Srinivasan, L., Calado, D. P., Patterson, H. C., Zhang, B., Wang, J., Henderson, J. M. et al.**, Lymphoproliferative disease and autoimmunity in mice with increased miR-17-92 expression in lymphocytes. *Nat. Immunol.* 2008. **9**: 405–414.

43 **Zhang, H., Li, Y., Huang, Q., Ren, X., Hu, H., Sheng, H. and Lai, M.**, MiR-148a promotes apoptosis by targeting Bcl-2 in colorectal cancer. *Cell. Death Differ.* 2011. **18**: 1702–1710.

44 **Jorgensen, T. N., McKee, A., Wang, M., Kushnir, E., White, J., Refaeli, Y., Kappler, J. W. et al.**, Bim and Bcl-2 mutually affect the expression of the other in T cells. *J. Immunol.* 2007. **179**: 3417–3424.

45 **Trotman, L. C., Alimonti, A., Scaglioni, P. P., Koutcher, J. A., Cordon-Cardo, C. and Pandolfi, P. P.**, Identification of a tumour suppressor network opposing nuclear Akt function. *Nature* 2006. **441**: 523–527.

46 **Haftmann, C., Stittrich, A. B., Sgouroudis, E., Matz, M., Chang, H. D., Radbruch, A. and Mashreghi, M. F.**, Lymphocyte signaling: regulation of FoxO transcription factors by microRNAs. *Ann. N Y Acad. Sci.* 2012. **1247**: 46–55.

47 **Tsukumo, S. I., Unno, M., Muto, A., Takeuchi, A., Kometani, K., Kurosaki, T., Igarashi, K. et al.**, Bach2 maintains T cells in a naive state by suppressing effector memory-related genes. *Proc. Natl. Acad. Sci. U S A* 2013. **110**: 10735–10740.

48 **Chang, H. D., Helbig, C., Tykocinski, L., Kreher, S., Koeck, J., Niesner, U. and Radbruch, A.**, Expression of IL-10 in Th memory lymphocytes is conditional on IL-12 or IL-4, unless the IL-10 gene is imprinted by GATA-3. *Eur. J. Immunol.* 2007. **37**: 807–817.

49 **Mashreghi, M. F.**, **Klemz, R.**, **Knosalla, I. S.**, **Gerstmayer, B.**, **Janssen, U.**, **Buelow, R.**, **Jozkowicz, A. et al.**, Inhibition of dendritic cell maturation and function is independent of heme oxygenase 1 but requires the activation of STAT3. *J. Immunol.* 2008. **180**: 7919–7930.

50 **Manz, R.**, **Assenmacher, M.**, **Pfluger, E.**, **Miltenyi, S. and Radbruch, A.**, Analysis and sorting of live cells according to secreted molecules, relocated to a cell-surface affinity matrix. *Proc. Natl. Acad. Sci. U S A* 1995. **92**: 1921–1925.

51 **Brett, S. J.**, **Baxter, G.**, **Cooper, H.**, **Rowan, W.**, **Regan, T.**, **Tite, J. and Rapson, N.**, Emergence of CD52-, glycosylphosphatidylinositol-anchor-deficient lymphocytes in rheumatoid arthritis patients following Campath-1H treatment. *Int. Immunol.* 1996. **8**: 325–334.

52 **Axtell, R. C.**, **de Jong, B. A.**, **Boniface, K.**, **van der Voort, L. F.**, **Bhat, R.**, **De Sarno, P.**, **Naves, R. et al.**, T helper type 1 and 17 cells determine efficacy of interferon-beta in multiple sclerosis and experimental encephalomyelitis. *Nat. Med.* 2010. **16**: 406–412.

53 **Quah, B. J.**, **Warren, H. S. and Parish, C. R.**, Monitoring lymphocyte proliferation in vitro and in vivo with the intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester. *Nat. Protoc.* 2007. **2**: 2049–2056.

Abbreviations: **Bcl2**: B-cell lymphoma 2 · **Bim**: Bcl2-interacting mediator of cell death · **HPRT**: hypoxanthine guanine phosphoribosyltransferase · **LCMV**: lymphocytic choriomeningitis virus · **miR/miRNA**: microRNA · **RAB11B**: Ras-related protein Rab-11B · **S1Pr1**: Sphingosine-1-phosphate receptor 1

Full correspondence: Dr. Mir-Farzin Mashreghi, Deutsches Rheuma-Forschungszentrum Berlin, Chariteplatz 1, 10115 Berlin
Fax: +49-30-28560603
e-mail: mashreghi@drfz.de

Current address: Anna-Barbara, Stittrich, Institute for Systems Biology, Seattle, WA 98109, USA

Current address: Gitta Aheinz, Institute of Molecular Immunology, Helmholtz Zentrum München, Munich, Germany

See accompanying article:
http://dx.doi.org/10.1002/eji.201444637

Received: 7/3/2014
Revised: 3/11/2014
Accepted: 2/12/2014
Accepted article online: 8/12/2014

3.7 Hopx fördert das Überleben von Th1-Zellen

Albrecht I, Niesner U, Janke M, Menning A, Loddenkemper C, Kühl AA, Lepenies I, Lexberg MH, Westendorf K, Hradilkova K, Grün J, Hamann A, Epstein JA, **Chang HD**, Tokoyoda K, Radbruch A. 2010. Persistence of effector memory Th1 cells is regulated by Hopx. *Eur J Immunol.* 40(11):2993-3006.

In physiologischen Immunantworten, durchläuft eine aktivierte und expandierte Th-Zellpopulation eine Kontraktionsphase, um die nicht mehr gebrauchten Th-Zellen zu eliminieren und Immunpathologie einzuschränken. Ein wichtiger Signalweg, der die Kontraktion von Th-Zellen fördert, wird über die Interaktion von Fas mit FasL (Fas-Ligand) geregelt. Wir haben in mehrfach restimulierten Th1 Zellen eine Hochregulation der Expression des Gens *Hopx* identifiziert. Ähnlich wie *Twist1* zeigte *Hopx* eine stetige Hochregulation in seiner Expression mit jeder weiteren Restimulation der Th1-Zelle. Wir konnten zeigen, dass Hopx durch den Transkriptionsfaktor T-bet reguliert wird und daher auch nicht in Th2 oder Th17 Zellen exprimiert wird.

Mäuse in denen das *Hopx*-Gen deletiert wurde zeigten eine selektive Reduktion IFNγ-produzierender Th- Zellen. Der Vergleich der Transkriptome von WT und Hopx-defizienten Th1-Zellen deutete darauf hin, dass Hopx eine Rolle bei der Apoptoseregulation spielt. Tatsächlich zeigten Hopx-exprimierende Th1-Zellen eine erhöhte Resistenz gegenüber Fas/FasL-induzierter Apoptose im Vergleich zu Hopx-defizienten Th1-Zellen. Um die physiologische Relevanz der Hopx-Expression von Th1-Zellen zu testen, haben wir die Hopx-Expression mittels shRNA in Th1-Zellen inhibiert und deren Kompetenz eine Entzündung auszulösen getestet. Im T-Zelltransfer-Kolitis Modell waren Th1-Zellen, in denen Hopx inhibiert wurde, nach dem adoptiven Transfer in Rag-defiziente Mäuse nicht in der Lage zu persistieren und konnten demnach auch keine Darmentzündung auslösen. Diese Ergebnisse zeigten, dass Hopx eine wichtige Rolle für das Überleben von pro-inflammatorischen Th1-Zellen *in vivo* spielt. Über welchen molekularen Mechanismus Hopx seine Funktion ausübt ist noch nicht bekannt. Hopx selbst hat keine DNA-bindende Aktivität und neuere Ergebnisse deuten darauf hin, dass Hopx im Zytosol lokalisiert ist. Mit welchen Faktoren Hopx in der Zelle interagiert wird zurzeit von uns untersucht.

European Journal of Immunology

Frontline

Persistence of effector memory Th1 cells is regulated by *Hopx*

Inka Albrecht[1], Uwe Niesner[1], Marko Janke[1], Astrid Menning[2], Christoph Loddenkemper[3], Anja A. Kühl[3], Inga Lepenies[1], Maria H. Lexberg[1], Kerstin Westendorf[1], Kristyna Hradilkova[1], Joachim Grün[1], Alf Hamann[2], Jonathan A. Epstein[4], Hyun-Dong Chang[1], Koji Tokoyoda[1,5] and Andreas Radbruch[1]

[1] German Rheumatism Research Center Berlin, Berlin, Germany
[2] Department of Rheumatology and Clinical Immunology of Charité Medical School, Berlin, Germany
[3] Department of Pathology/RCIS, Charité Medical School, Campus Benjamin Franklin, Berlin, Germany
[4] Department of Medicine, University of Pennsylvania Health System, Philadelphia, PA, USA
[5] Department of Immunology, Graduate School of Medicine, Chiba University, Chiba, Japan

Th1 cells are prominent in inflamed tissue, survive conventional immunosuppression, and are believed to play a pivotal role in driving chronic inflammation. Here, we identify *homeobox only protein* (Hopx) as a critical and selective regulator of the survival of Th1 effector/memory cells, both *in vitro* and *in vivo*. Expression of *Hopx* is induced by T-bet and increases upon repeated antigenic restimulation of Th1 cells. Accordingly, the expression of *Hopx* is low in peripheral, naïve Th cells, but highly up-regulated in terminally differentiated effector/memory Th1 cells of healthy human donors. In murine Th1 cells, Hopx regulates the expression of genes involved in regulation of apoptosis and survival and makes them refractory to Fas-induced apoptosis. *In vivo*, adoptively transferred Hopx-deficient murine Th1 cells do not persist. Consequently, they cannot induce chronic inflammation in murine models of transfer-induced colitis and arthritis, demonstrating a key role of Hopx for Th1-mediated immunopathology.

Key words: Cell survival · CD4 T cells · Inflammation · Memory cells

Supporting Information available online

Introduction

T-helper type 1 (Th1) cells mediate immune responses to intracellular pathogens, such as viruses, and produce IFN-γ as their signature cytokine [1]. IFN-γ, together with IL-12 and the transcription factors STAT1, STAT4 and T-bet, promotes the development of Th1 cells [2–3]. T-bet (T-box 21) is considered to act as the master transcription factor critically regulating Th1 lineage commitment [3–5]. Apart from their protective role in clearing infections, Th1 cells can initiate and maintain chronic inflammatory diseases, *e.g.* inflammatory bowel disease [6–9], uveitis [10], EAE [11, 12] and arthritis [13]. *In vitro*, Th1 cells are much more sensitive to Fas-mediated apoptosis than Th2 or Th17 cells [14–20]. *In vivo*, however, effector/memory Th1 cells are abundant in chronically inflamed tissue [21–23] and persist over long time periods [24–26], suggesting that their sensitivity to

Correspondence: Prof. Andreas Radbruch
e-mail: radbruch@drfz.de

Fas-mediated apoptosis is strictly regulated. Here, we demonstrate that among $CD4^+$ T cells, the transcriptional cofactor *homeobox only protein* (*Hopx*) is expressed by repeatedly restimulated Th1 cells, but not by Th2, Th17 or regulatory T cells. Hopx regulates Fas-mediated apoptosis of effector/memory Th1 cells and is critically required for their persistence *in vivo*.

In vertebrates, *Hopx* expression originally was detected in the myocardium [27, 28]. There, expression of *Hopx* is induced by the cardiac transcription factor Nkx2-5. Hopx-deficient mice show a complex, incompletely penetrant phenotype. Some Hopx-deficient embryos have a poorly developed myocardium with reduced cell numbers, others show normal, and still others show increased numbers of cardiomyocytes after birth [27, 28]. Hopx does not bind to homeobox consensus binding sequences, and it has been postulated that Hopx acts indirectly, partnering with other transcription factors [27, 28]. In cardiomyocytes, Hopx regulates the expression of specific genes, by repressing serum response factor (SRF)-induced transcription [27, 28]. Although *Hopx* is expressed not only in cardiomyocytes, but also in many other cell types and tissues, Hopx-deficient mice have no apparent phenotype other than cardiac disorders [27, 28].

Here, we show that in Th lymphocytes, the Th1 transcription factor T-bet induces expression of *Hopx*. *Hopx* expression is specific for resting Th1 cells, and is up-regulated upon each subsequent restimulation. Hopx-deficient mice show a significant decrease in overall numbers of effector/memory Th1 cells. Hopx-deficient Th1 cells cannot induce chronic inflammation, *e.g.* murine transfer-induced colitis or arthritis, and show a decreased persistence *in vivo* and *in vitro*, demonstrating the pivotal role of Hopx for Th1-mediated immunopathology.

Results

Hopx is expressed by Th1, but not by Th2 or Th17 cells

To determine whether *Hopx* is expressed in Th1 cells, we compared the transcriptomes of Th1 cells stimulated with antigen either once or four times *in vitro*. Naïve $CD4^+CD62L^+$ Th lymphocytes expressing the transgenic DO11.10 TCR specific for OVA were isolated and activated for 6 days with the cognate peptide $OVA_{327–339}$ and splenic APC. Functional differentiation into Th1 cells was achieved by adding IL-12. Cells were restimulated every 6 days for up to four cycles, and gene expression profiles of cells stimulated once or four times were compared as described previously [29]. *Hopx* was expressed in Th1 cells, and its expression was up-regulated in Th1 cells that had been repeatedly restimulated (Supporting Information Fig. 1).

Expression of *Hopx* in Th1 cells was validated at the mRNA and protein levels and compared with distinct Th effector cell lineages by quantitative PCR and immunoblot. *Hopx* expression in Th2 and Th17 cells was low, at the limit of detection (Fig. 1A and B; Supporting Information Fig. 2). *Hopx* expression was selectively up-regulated by about 20-fold at the mRNA and protein levels in Th1 cells. In Th1 cells, *Hopx* expression correlated with the number

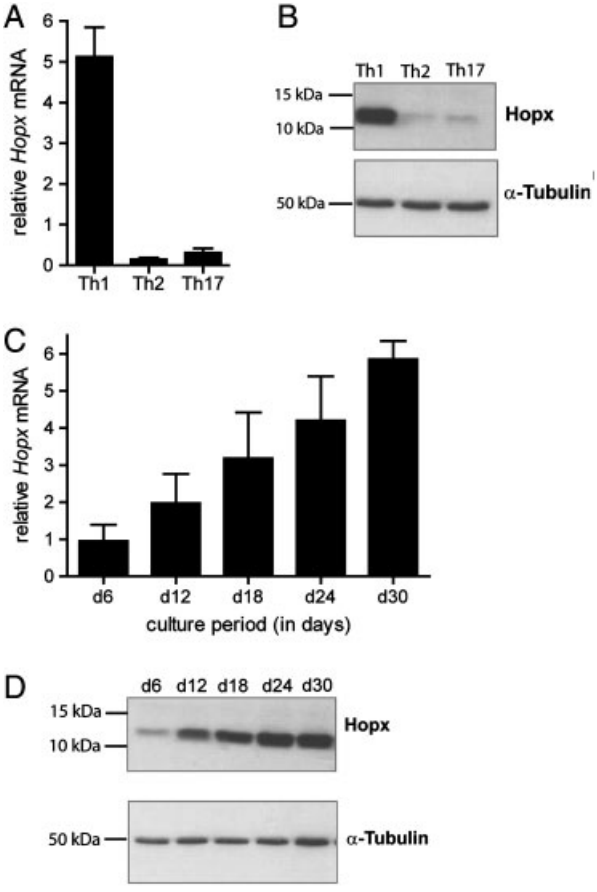


Figure 1. Hopx expression characterizes Th1 cells and correlates with the number of antigenic activations. (A) $CD4^+CD62L^+$ OVA-specific T cells were stimulated *in vitro* for four cycles (24 days) under Th1, Th2, or Th17 polarizing conditions. Functional polarization of Th1, Th2, and Th17 cells, *i.e.* the cytokine expression profile, was confirmed by intracellular cytokine staining (Supporting Information Fig. 2). Expression of *Hopx* mRNA in resting cells was determined by RT-PCR and normalized to *hypoxanthine guanine phosphoribosyl transferase*. Results represent the mean+SD of three independent experiments. (B) Hopx protein expression in resting 24-day-old Th1, Th2, and Th17 cells. Control: α-tubulin immunoblot. One representative immunoblot out of the two independent experiments is shown. (C) *Hopx* mRNA expression in repeatedly activated Th1 cells was determined every 6 days (mean+SD of three independent experiments). (D) Hopx protein expression, corresponding to the mRNA experiment shown in (C), as determined by immunoblot. The data are representative of three independent experiments.

of antigenic reactivations, and was up-regulated fourfold when comparing cells stimulated once with those stimulated four times (Fig. 1C and D). *Hopx* mRNA and protein levels reached a maximum after five cycles of activation for DO11.10 Th1 cells (Fig. 1C and D), or two cycles of activation for OVA TCR-specific OT2 Th1 cells (data not shown), and remained stable thereafter.

Hopx expression is induced by T-bet

The transcription factor T-bet has been shown to induce expression of *ifn-γ*, and is considered to be the master transcription factor of

Th1 differentiation [3]. Here, we show that T-bet is required for induction of *Hopx* expression. T-bet$^{-/-}$ Th cells, when polarized with IFN-γ and IL-12, do not express *Hopx*, suggesting that Stat1 and Stat4 are insufficient to induce *Hopx* expression, whereas they are sufficient to induce expression of *ifn-γ*, albeit at reduced levels as is evident when comparing IFN-γ levels between Tbet$^{-/-}$ Th1 polarized cells with their WT counterparts (Fig. 2A and B). Conversely, retroviral overexpression of T-bet induces expression of *Hopx* in activated Th cells, even in the absence of IL-12 and IFN-γ (Supporting Information Fig. 3A and B).

Neither Stat1 nor Stat4 signaling appears to be involved in induction of *Hopx* expression (Fig. 2C–F). In IFN-γR$^{-/-}$ Th1 cells, expectedly showing reduced IFN-γ production (Fig. 2C), expression of *Hopx* was comparable to WT Th1 cells (black bars, Fig. 2D). Moreover, we measured *T-bet* mRNA expression and observed that the level of induction of *Hopx* and *T-bet* transcripts correlated in all cell populations analyzed (grey bars, Fig. 2D). STAT4$^{-/-}$ Th cells activated in the presence of IL-12 and IFN-γ were impaired in their differentiation into Th1 cells (Fig. 2E), and in the expression of T-bet (Fig. 2F), as expected. However, the levels of induction of *Hopx* and *T-bet* mRNA were comparable in STAT4$^{-/-}$ Th1 cells, and *Hopx* expression was induced threefold in STAT4$^{-/-}$ Th1 cells, as compared with STAT4$^{-/-}$ Th2 cells (Fig. 2F).

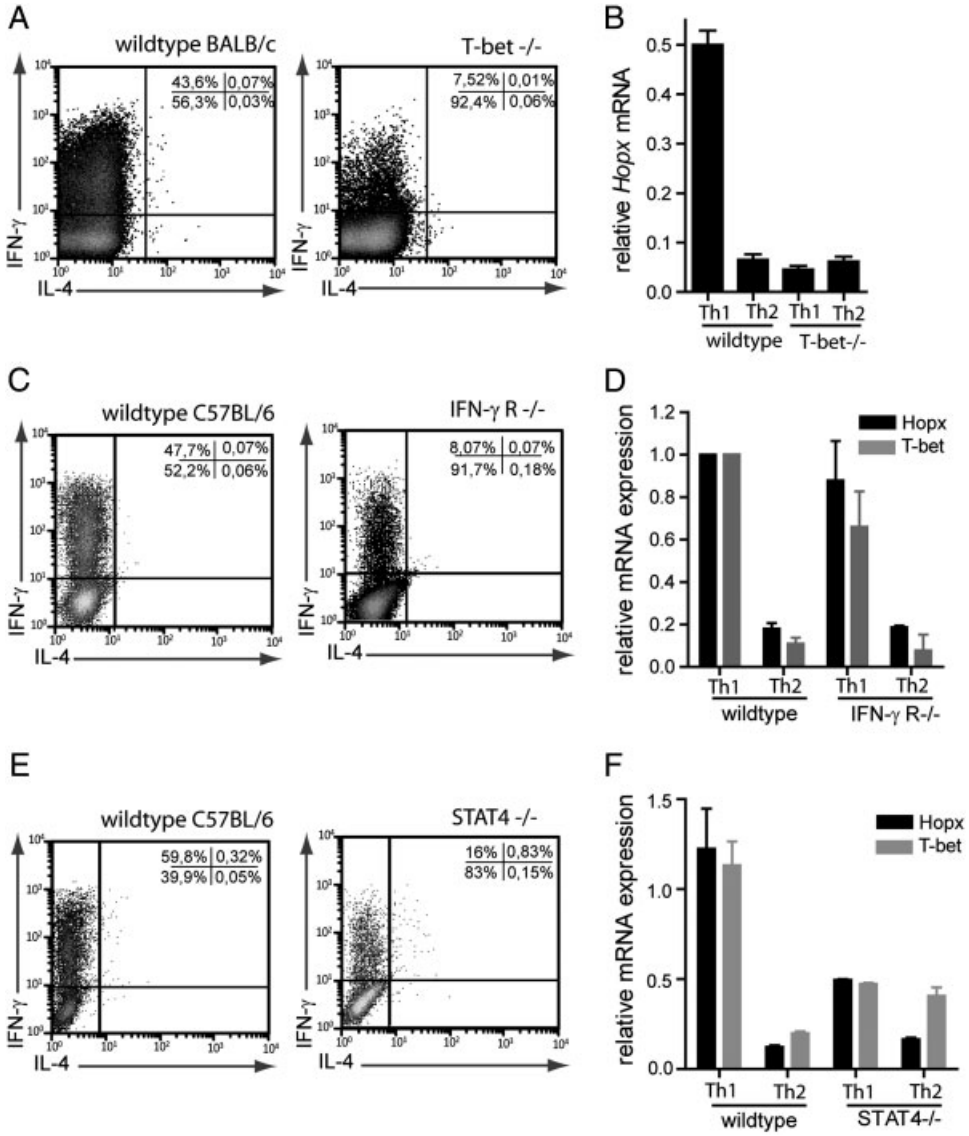


Figure 2. *Hopx* is induced by the Th1 master transcription factor T-bet. (A and B) CD4$^+$CD62L$^+$ Th cells of T-bet$^{-/-}$ and congenic BALB/c mice were stimulated with anti-CD3/anti-CD28 under Th1 (IL-12 and IFN-γ) or Th2 (IL-4)-polarizing conditions for 6 days. (A) IFN-γ expression was measured by intracellular cytokine staining. (B) *Hopx* mRNA was quantified by RT- PCR. (C–F) Naïve Th cells were isolated from (C, D) IFN-γR$^{-/-}$ or (E, F) STAT4$^{-/-}$ as well as congenic WT mice and activated with anti-CD3/CD28 under Th1 or Th2-polarizing conditions. Representative cytokine staining of (C) IFN-γR$^{-/-}$ or (E) STAT4$^{-/-}$ Th1 compared with congenic Th1 cells. *Hopx* (black bars) and *T-bet* (grey bars) mRNA expression measured in (D) IFN-γR$^{-/-}$ or (F) STAT4$^{-/-}$ as compared with WT Th cells on day 6. Results represent the mean+SD (mRNA expression data) or are representative of two to three independent experiments (cytokine expression).

Hopx is a marker of effector/memory Th1 cells

In order to determine whether expression of *Hopx* follows the differentiation of naïve Th cells into advanced effector/memory phenotype Th cells *in vivo*, $CD4^+$ Th cells from aged C57BL/6 mice were sorted according to their expression of CD44 and CD62L into naïve ($CD44^-CD62L^+$), central memory (CM, $CD44^+CD62L^+$), and effector memory (EM, $CD44^+CD62L^-$) Th cells. *Hopx* transcript levels were low in naïve Th cells and in CM Th cells (Fig. 3A). Compared with naïve $CD4^+$ T cells, *Hopx* was up-regulated by 4.5-fold in EM Th cells, reaching expression levels comparable to those of repeatedly *in vitro* restimulated Th1 cells.

We also investigated whether *Hopx* is expressed in human Th cells resembling repeatedly restimulated effector/memory cells (Fig. 3B). In naïve ($CD45RA^+CCR7^+$) and in CM ($CD45RA^-CCR7^+$) Th cells [30], *Hopx* was expressed at low but detectable levels. In EM ($CD45RA^-CCR7^-$) Th cells [30], *Hopx* expression was up-regulated 18-fold, as compared with naïve Th cells. In "terminally differentiated" EM ($CD45RA^-CCR7^-CD27^-$) Th cells [31, 32], *Hopx* was up-regulated 38-fold, and 45-fold in EM ($CD45RA^-CCR7^-CCR5^+$) Th1 cells [33], again in comparison to naïve Th cells. In regulatory ($CD25^{hi}$ $CD127^-$) Th cells [34] and in Th2 EM ($CD45RA^-CCR7^-CRTh2^+$) (CRTh2, chemoattractant receptor-homologous molecule expressed on Th2 cells) cells [35] (Fig. 3B), *Hopx* expression was not significantly up-regulated. Thus, *Hopx* expression marks human Th1 cells *in vivo*, and its expression is up-regulated in Th1 cells which are presumably "experienced".

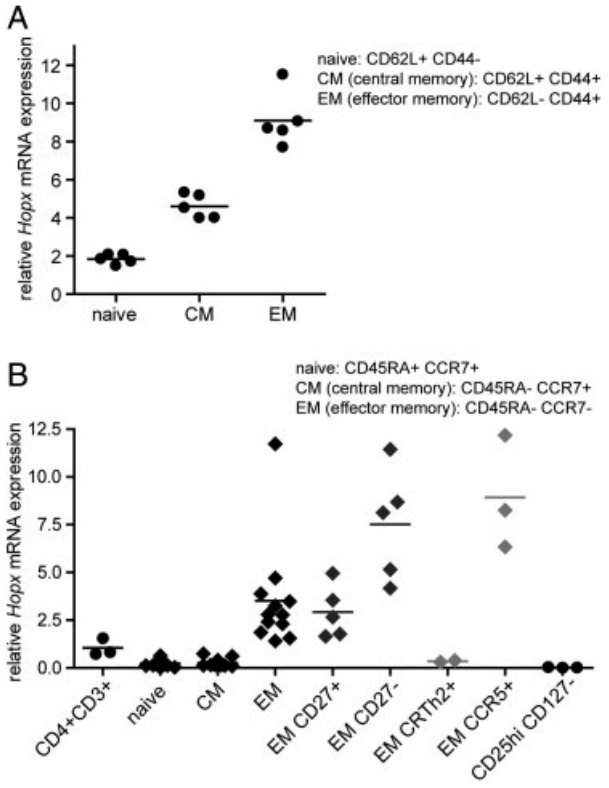


Figure 3. Hopx is a marker of effector/memory Th1 cells. (A) Splenic cells were isolated from untreated, 5-month-old C57BL/6 mice and sorted into different subpopulations according to their expression of CD4, CD62L, and CD44. *Hopx* mRNA was quantified by RT-PCR in each subpopulation. Each data point represents one individual mouse ($n = 5$ for each group), and horizontal bars indicate mean values. (B) *Hopx* mRNA was quantified in peripheral human T lymphocytes. The expression of *Hopx* mRNA normalized to *ubiquitin ligase H5* in total $CD4^+CD3^+$ T cells ($n = 3$) was set to 1. Subpopulations were defined according to expression of the following surface markers: naïve ($CD4^+$ $CD45RA^+CCR7^+$) ($n = 17$), CM ($CD4^+CD45RA^-CCR7^+$) ($n = 19$), EM ($CD4^+$ $CD45RA^-CCR7^-$) ($n = 12$), EM $CD27^-$, EM $CD27^+$ (each $n = 5$), EM CRTh2 representing Th2 EM T cells ($n = 2$), EM $CCR5^+$ considered to be Th1 EM T cells ($n = 3$), and regulatory $CD25^{hi}CD127^-$ T cells ($n = 3$) with data points representing different individual healthy donors and horizontal bars indicating the mean.

Hopx does not influence development of Th1 cells, but their persistence

To investigate the function of *Hopx* in Th1 cells, we analyzed the influence of *Hopx* on the induction and polarization of Th1 cells and their persistence. Naïve Hopx-deficient TCR transgenic Th cells were activated twice with their cognate antigen *in vitro*, under Th1 or Th2 polarizing conditions. After 2 wk, the frequencies of IFN-γ-expressing Th1 and IL-4-expressing Th2 cells in cultures of $Hopx^{-/-}$, $Hopx^{+/-}$, and $Hopx^{+/+}$ OT2 cells were equivalent (Fig. 4A), demonstrating that *Hopx* does not detectably influence the induction of Th1 or Th2 cells. However, *Hopx* does impact on the persistence of Th1 cells *in vivo*. As compared with $Hopx^{+/-}$ and WT mice, $Hopx^{-/-}$ mice had significantly fewer Th1 effector/memory cells in their spleens and lymph nodes, as identified by fast expression of IFN-γ (Fig. 4B and C). Although Th1 effector/memory cells are reduced in frequencies, IL-17 expressing $CD4^+$ T cells were not affected (Fig. 4B and C), showing that the impact of *Hopx* is Th1-specific. The genetic deletion of *Hopx* also does not impact on the absolute number of $CD4^+$ T cells (Supporting Information Fig. 4).

To determine whether the effect of *Hopx* on Th cell persistence *in vivo* is due to *Hopx* expressed by Th1 cells, Hopx-deficient Th1 cells were generated and adoptively transferred into WT mice. To generate the cells, naïve OT2 Th cells were activated twice *in vitro* with cognate antigen and polarized into Th1 cells. Expression of *Hopx* in these cells was knocked down by a *Hopx*-targeting small hairpin RNA (shRNA). *Hopx*-targeting shRNA reduced the level of endogenous *Hopx* expression in Th1 cells by approximately 90–95% at the mRNA level (Fig. 4D, left). A strong down-modulation of Hopx by the *Hopx*-targeting shRNA was also detected at the protein level (Fig. 4D, middle). Hopx-deficient and Hopx-competent Th1 cells cultured for 12 days were adoptively transferred into naïve, congenic C57BL/6 mice. 21 days later, the spleen was examined for persistent OT2 Th1 cells. The persistence of Th1 cells with knocked down *Hopx* expression was significantly reduced to about 50%, as compared with controls (Fig. 4D, right).

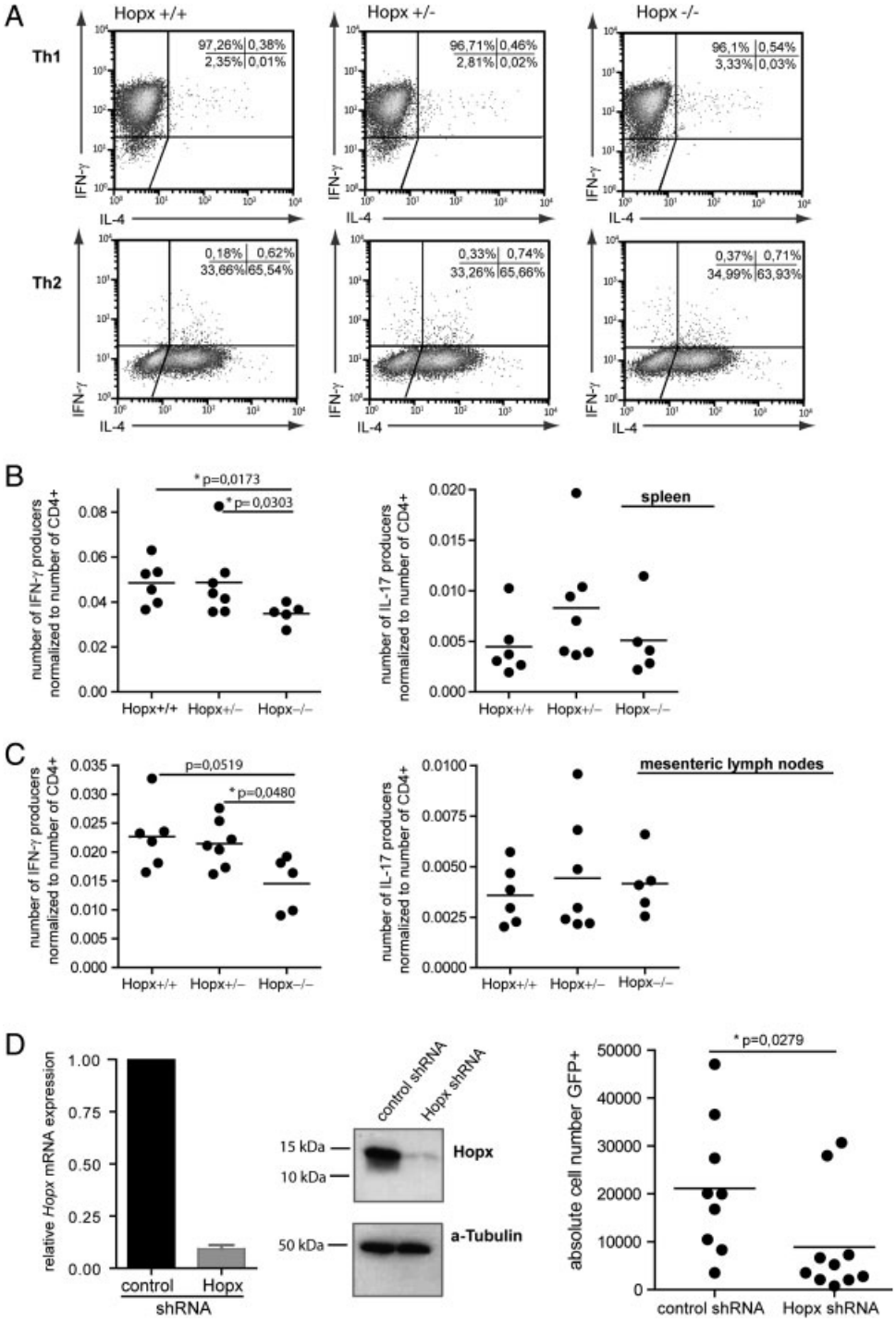


Figure 4. Hopx promotes persistence of Th1 cells, but does not influence Th1 polarization. (A) Naïve OT2 $CD4^+$ T cells of $Hopx^{+/+}$, $Hopx^{+/-}$, and $Hopx^{-/-}$ mice were stimulated twice with OVA peptide under Th1- or Th2-polarizing conditions. On day 12, cells were restimulated and stained for intracellular expression of IFN-γ and IL-4. Data are representative of five independent experiments. (B and C) $CD4^+$ T cells from aged Hopx-deficient and control mice were analyzed by intracellular staining for the frequency of Th1 and Th17 cells following short-term mitogenic PMA/Ionomycin stimulation in the presence of Brefeldin A. Frequencies were determined by counting the absolute cell numbers of IFN-γ and IL-17 producing $CD4^+$ T cells in the (B) spleen and (C) mesenteric lymph nodes of $Hopx^{-/-}$ ($n = 5$), $Hopx^{+/-}$ ($n = 7$), and $Hopx^{+/+}$ ($n = 6$) mice using FlowCount beads and calculating the ratio to cell numbers of $CD4^+$ T cells. Each data point represents one individual mouse and horizontal bars are the means. The result is representative for two independent experiments. (D) OT2 Th1 cells infected with a retrovirus encoding a *Hopx*-targeting shRNA or a non-sequence-specific control shRNA and stimulated twice were sorted for expression of the viral marker gene *gfp* and checked for Hopx mRNA (left) and protein expression levels (middle). On day 12, viable GFP^+ Th1 cells were adoptively transferred into congenic C57BL/6 mice. The absolute cell numbers of GFP^+ Th1 cells in the spleens of the recipient mice were determined 21 days after transfer by flow cytometry using FlowCount beads (right). The data shown are the mean of two independent experiments each performed with four to five mice *per* group. Data points represent the result for the individual mice (control shRNA: $n = 9$, Hopx shRNA $n = 10$). Horizontal bars are the means. Statistics: two-tailed Mann–Whitney test.

Hopx regulates expression of apoptotic genes and lowers sensitivity to Fas-mediated apoptosis

The effect of *Hopx* on the persistence of Th1 cells *in vivo* could be due to regulation of either proliferation or apoptosis. *In vitro*, Hopx$^{-/-}$ Th1 cells did not show a defect in proliferation upon activation with antigen (Supporting Information Fig. 5), indicating that it was not the former. To investigate the latter, after two antigenic stimulations, and 6 days of rest, viable Th1 cells were treated with 2 μg/mL of an agonistic Fas-antibody (Jo2) to induce apoptosis. In brief, 12 h later, 20 or 7.7% of Hopx$^{-/-}$ Th1 cells were annexin V-positive or propidium iodide-annexin V-double positive, respectively, as compared with 11 or 5.9% of WT controls, respectively (Fig. 5A). In total, 72 h later, the numbers of viable Hopx$^{-/-}$ Th1 cells were 40–50% lower than those of heterozygous and WT controls (Fig. 5B); untreated Th1 cells did not show any differences (Fig. 5A and B). Thus Hopx-deficient and Hopx-competent Th1 cells differed in their susceptibility to Fas-mediated apoptosis.

To characterize the mechanism of action of Hopx in more detail, we compared the transcriptomes of Hopx$^{-/-}$ Th1 cells to those of Hopx$^{+/-}$ and Hopx$^{+/+}$ Th1 cells. The cells were restimulated twice and dead cells were removed by gradient centrifugation. Briefly, 54 genes were differentially expressed by a factor of 1.3 or more when comparing Hopx$^{-/-}$ *versus* Hopx$^{+/+}$ Th1 cells (Supporting Information Fig. 6). A substantial number of those genes are known to be involved in the regulation of cell survival. In agreement with the anti-apoptotic role of Hopx in Th1 cells (Fig. 5), expression of anti-apoptotic genes was up-regulated by Hopx, whereas expression of pro-apoptotic genes was down-regulated. Among the anti-apoptotic genes positively regulated by Hopx and consequently down-regulated in Hopx$^{-/-}$ Th1 cells was *Atp8a1* (APLT) encoding for an aminophospholipid-transporting enzyme important for maintaining phospholipid asymmetry and thus integrity of the cell membrane [36–40]. Expression of *Nrn1* (neuritin 1) was also reduced in Hopx-deficient Th1 cells. Nrn1 has been described to protect cells from apoptosis, by inhibiting activation of caspase 3 [41]. Further anti-apoptotic genes with reduced activity in Hopx$^{-/-}$ Th1 cells were *Bcl11b* [42], *Gna13* (Galpha13) [43] and *Hsp90ab1* [44, 45] (Supporting Information Fig. 6). Pro-apoptotic genes were down-regulated by Hopx, *e.g.* the lysosome-associated apoptosis-inducing protein *Plekhf1* [46, 47], and *Sox4* (SRY-box containing gene 4), a sensor for DNA damage [48]. Together, the transcriptome analysis suggests that besides Fas-mediated apoptosis other death pathways could also be influenced by Hopx.

Hopx-deficient Th1 cells fail to induce chronic inflammation

The Th1-specific expression of *Hopx*, its up-regulation in repeatedly restimulated Th1 cells, and its antagonism to Fas-induced apoptosis suggest an essential role for *Hopx* in regulating Th1-induced chronic immune reactions and immunopathology.

To investigate this, Th1 cells were intravenously injected into Rag1$^{-/-}$ mice to induce colitis [49]. The Th1 cells had been generated from C57BL/6 naïve Th cells, and activated with anti-CD3 and anti-CD28 in the presence of IL-12 for 6 days. Endogenous *Hopx* expression by the Th1 cells was knocked down by a specific

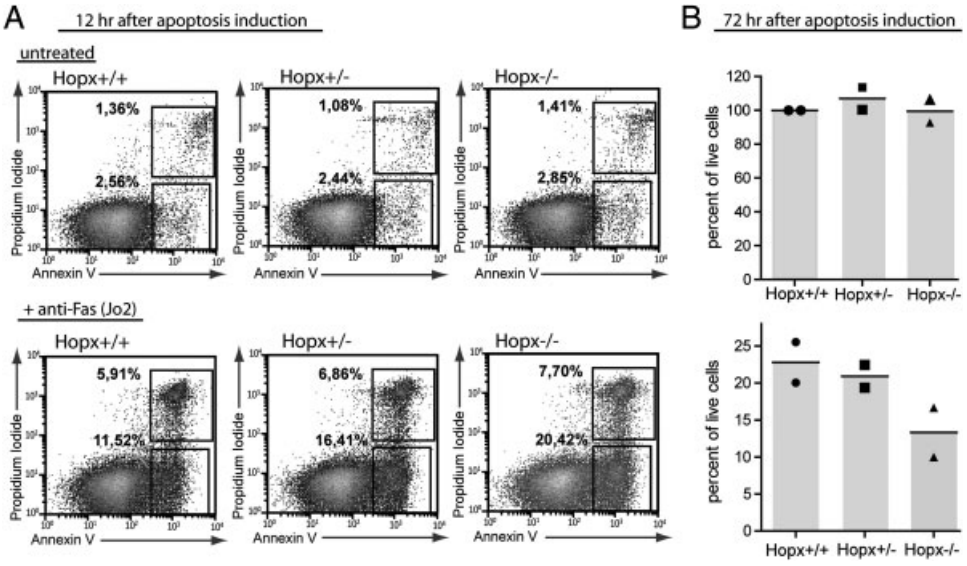


Figure 5. Hopx$^{-/-}$ Th1 cells display increased susceptibility to Fas-mediated apoptosis. (A) Naïve Hopx$^{-/-}$, Hopx$^{+/-}$, and Hopx$^{+/+}$ OT2 Th cells were activated twice with OVA peptide under Th1-polarizing conditions. On day 12, viable cells were harvested and apoptosis was induced by adding an agonistic Fas antibody (Jo2) or the cells were left untreated. Briefly, 12 h after apoptosis induction, the cells were stained with annexin V and propidium iodide. Percentages of annexin V$^+$ and annexin V$^+$propidium iodide$^+$ double positives are indicated. (B) In total, 72 h after apoptosis induction, the viable cells were counted after propidium iodide exclusion by flow cytometry using FlowCount beads. The numbers of viable Hopx$^{+/+}$ Th1 cells without treatment was set to 100% and the percentages of viable cells in the other groups were calculated accordingly. Data are representative of two independent experiments (A) or represent the individual results and mean (B).

shRNA. Seven weeks after transfer, mice that had received control Th1 cells developed diarrhea and gradually lost weight (Supporting Information Fig. 7), whereas mice that had received Th1 cells with knocked down *Hopx* expression did not show these symptoms. In mice which had been reconstituted with control Th1 cells, histological analysis of the colon 8–9 wk after transfer revealed severe inflammation with maximal scores (Fig. 6A), with a complete loss of the normal crypt architecture, massive infiltration of cells in the lamina propria and submucosa, loss of goblet cells, crypt abscess formation, and massive epithelial hyperplasia (Fig. 6C). The colons of mice which had received Hopx-knocked down Th1 cells showed either no or only weak inflammatory responses, with no or only minimal cell infiltration of the mucosa by leukocytes (Fig. 6A and C). The inflammatory scores correlated with the persistence of Th1 cells in the spleen and mesenteric lymph nodes of the host mice since the numbers of Hopx-deficient Th1 cells were reduced more than tenfold, both in spleen and in lymph nodes, as compared with control Th1 cells (Fig. 6B).

These results were confirmed in antigen-induced arthritis as a second model of chronic inflammation. $Hopx^{-/-}$, $Hopx^{+/-}$, and WT OT2 Th1 cells were adoptively transferred into $Rag1^{-/-}$ mice. The cells had been activated *in vitro* twice with OVA and IL-12, and, as expected, Hopx-competent Th1 cells highly expressed Hopx mRNA and protein, whereas Hopx-deficient Th1 cells did not (Fig. 6D). One day after transfer of the Th1 cells, arthritis was induced by injection of cationized OVA into the knee. The knees were histologically inspected 2 wk later. Although $Hopx^{+/+}$ and $Hopx^{+/-}$ Th1 cells induced inflammation with histological scores of 5–6, $Hopx^{-/-}$ Th1 cells induced histological inflammatory scores of about 3 (Fig. 6E) on average. Transfer of $Hopx^{-/-}$ Th1 cells led to reduced infiltration of mononuclear cells (Fig. 6F, left panel). Remarkably, few if any $Hopx^{-/-}$ Th1 cells persisted in the inflamed knees, as compared with $Hopx^{+/-}$ or $Hopx^{+/+}$ Th1 cells (Fig. 6F, right panel). The absolute numbers of $CD4^+$ T cells persisting in the spleen and draining popliteal lymph nodes did not differ between Hopx-competent and Hopx-deficient Th1 cell transfers (Supporting Information Fig. 8).

Discussion

The homeobox transcriptional cofactor *Hopx* was first described in 2002 [27, 28]. Hopx is expressed in developing cardiomyocytes regulating their differentiation and survival during embryogenesis. Meanwhile, expression of *Hopx* has been detected in several other tissues, *i.e.* the brain, lung, liver, skeletal muscle, intestine, and spleen [27, 28, 50]. Here, we describe that *Hopx* in the Th-cell lineage is expressed selectively by Th1 cells, not by Th2, Th17, or regulatory T cells. Expression of *Hopx* in Th1 cells incrementally increases upon each restimulation and is highest in terminally differentiated effector/memory Th1 cells. In Th1 cells, *Hopx* expression is induced by the transcription factor T-bet. Hopx-deficient Th1 cells show increased susceptibility to Fas-induced apoptosis, decreased persistence *in vivo*, and cannot induce chronic inflammation in murine models of arthritis or colitis. Thus, *Hopx* is an essential factor for the survival of Th1 effector/memory cells and their contribution to chronic immunopathology.

In embryonic development of cardiomyocytes, *Hopx* regulates the balance between expansion and differentiation of the myocytes by interfering with SRF-mediated transcription [27, 28]. Accordingly, several known SRF target genes are up-regulated in Hopx-deficient hearts [28]. Differentially expressed genes by a factor of ≥ 1.3 that were identified by gene expression profiling of Hopx-deficient *versus* Hopx-competent Th1 cells are not yet known to be targets of SRF-mediated transcription. However, several differentially expressed genes in Hopx-deficient Th1 cells, as compared with WT control cells, are known to be involved in the regulation of cell survival, a finding consistent with the increased apoptotic death rate of Hopx-deficient Th1 cells. In myocytes, Hopx can also interact with histone deacetylase 2 and recruits it to SRF–Hopx complexes [51]. Interestingly, histone deacetylase inhibitors have been reported to activate apoptotic pathways including Fas-mediated apoptosis [52–55] and exhibit therapeutic potential in the treatment of cancer [56, 57] and inflammatory diseases [58, 59]. In skeletal muscle cells, Hopx is expressed as well, but acts independently of SRF and mostly activates transcription of the target genes [60]. In those cells, enhancer of polycomb 1 has been identified as a novel interaction partner of Hopx [60]. In Th1 cells, Hopx regulates expression of a limited number of genes. Transcription of 54 genes is changed 1.3-fold or more between WT and Hopx-deficient Th1 cells. In total, 14 of these genes are down-regulated by Hopx and 40 genes up-regulated, demonstrating that Hopx is not merely acting as a transcriptional repressor in Th1 cells.

As we show here, expression of *Hopx* is induced in Th1 cells by the T-box transcription factor T-bet (Tbx-21). Neither TCR signals, nor IFN-γ or IL-12/27, *i.e.* Stat1 or Stat4 are required. Stat1 and Stat4, and the cytokines activating them, will be involved *in vivo*, however, as they regulate T-bet expression in Th1 cells, and the imprinting of Th1-specific genes [61]. *Hopx* obviously is imprinted for expression, since it is expressed at increasing levels upon subsequent restimulations. In cardiomyocytes, the homeobox transcription factor Nkx2-5 regulates expression of Hopx [27, 28]. Interestingly, the T-box transcription factors T-box 5 and 20 have been shown to form a functional complex with Nkx2-5 [62–64].

In vitro, *Hopx* is selectively expressed by effector/memory Th1 cells with increasing expression between subsequent restimulations. We could not detect expression in Th2 or Th17 cells, not even after repeated restimulation. In Th cells isolated *ex vivo*, expression of *Hopx* is restricted to presumptive Th1 effector/memory cells, as is evident from the analysis of human peripheral blood T cells. It is highest in $CCR7^-CD27^-$ "terminally differentiated" [31, 32] effector/memory Th cells and $CCR7^-CCR5^+$ effector/memory Th1 cells. Th2 effector/memory cells expressing CRTh2 express little if any *Hopx*, as do CCR7-expressing CM cells, naïve $CD4^+$ T cells ($CD45RA^+CCR7^+$) and regulatory T cells ($CD25^{hi}CD127^-$).

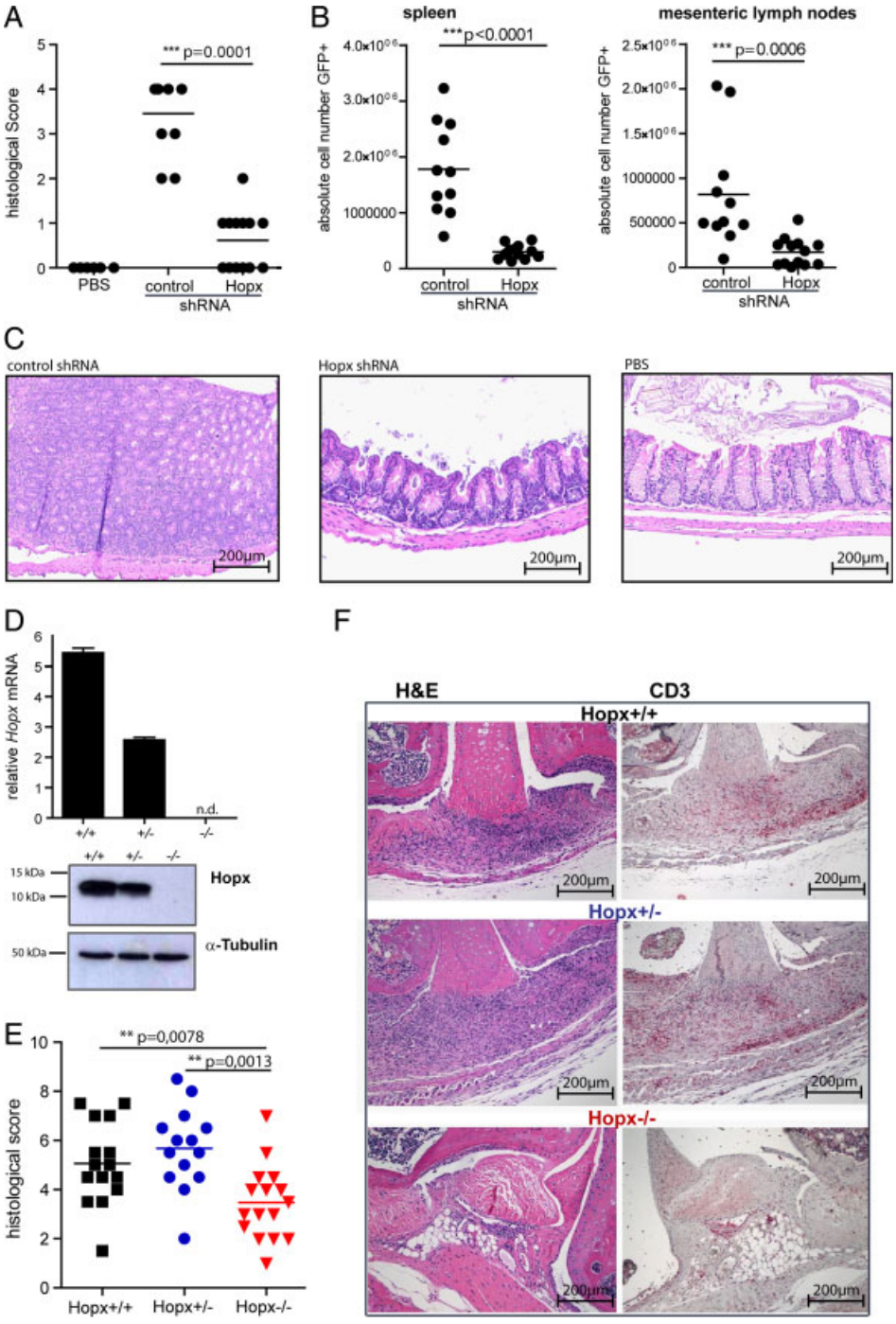


Figure 6. Hopx deletion results in a reduced capacity to induce inflammation in murine transfer colitis and antigen-induced arthritis. (A) Th1 cells stably expressing a retrovirus encoding for a Hopx-specific shRNA or a control shRNA and stimulated with anti-CD3/CD28 for 6 days were transferred i.v. into Rag1$^{-/-}$ mice. Totally, 8 to 9 wk after transfer, the colon was stained with hematoxylin/eosin and the severity of inflammation was scored. (B) Mice were analyzed for the persistence of transferred Th1 cells in the spleens and mesenteric lymph nodes by counting the number of GFP^{+} cells using FlowCount beads. The data shown are the mean of three independent experiments each performed with four to six mice *per* group and data points represent the result for the individual mice. Horizontal bars indicate the mean. (C) Hematoxylin/eosin staining of mice that received Hopx knocked down Th1 cells, control Th1 cells expressing a non-sequence-specific shRNA or PBS. The data are representative of PBS: $n = 6$, control shRNA: $n = 11$, Hopx shRNA $n = 13$ mice. (D) Hopx$^{-/-}$, Hopx$^{+/-}$, or Hopx$^{+/+}$ OT2 Th1 cells were activated twice with OVA antigen *in vitro* and analyzed for expression of Hopx mRNA and protein levels. The data are representative of two independent experiments. (E and F) The activated OT2 Th1 cells were adoptively transferred into Rag1$^{-/-}$ mice and arthritis was induced 24 h later by intra-articular injection of cationized OVA. In total, 2 wk after arthritis induction, the knee joints were histologically examined. (E) Histological scores from two independent experiments each performed with six to nine mice *per* group. The data points represent the results for the individual mice (transfer of Hopx$^{-/-}$ ($n = 16$), Hopx$^{+/-}$ ($n = 14$), and Hopx$^{+/+}$ ($n = 15$)); horizontal bars are the means. (F) Representative hematoxylin/eosin (left panel) and CD3 staining (right panel) of knee joint sections. Statistic analysis was performed by the two-tailed Mann–Whitney test.

In the division of labor between Th cell lineages, Th1 cells are responsible for acute and chronic inflammation and elimination of intracellular pathogens. Since they have to eliminate the cells hosting the pathogens, Th1 cells can also induce considerable immunopathology. In the case of persistent pathogens, the function and persistence of the specific Th1 cells have to be strictly regulated to limit their immunopathogenicity and maintain their (limited) protection. We have shown before that, to this end, repeatedly restimulated Th1 cells express distinct genes of high functional relevance, genes not expressed by Th2, Th17 or regulatory T cells. One such gene is *twist1*, a gene limiting the inflammatory potential of repeatedly restimulated Th1 cells [29]. Expression of *twist1* is induced by concomitant signaling of the TCR and Stat4, is transient upon TCR signaling, and incrementally increases upon repeated restimulation. As an E-box binding transcription factor, Twist1 forms a negative feedback loop with NF-κB and regulates the expression of pro-inflammatory cytokines. Here, we describe a second gene selectively expressed by Th1 cells, among Th cells, namely *Hopx*. As a homeobox transcriptional cofactor, *Hopx* controls persistence of repeatedly restimulated Th1 cells in between restimulations, by increasing their resistance to Fas-mediated apoptosis.

Hopx$^{-/-}$ mice have significantly reduced frequencies of IFN-γ^+ effector/memory Th cells in the spleen and lymph nodes, when compared with heterozygous or WT littermates, whereas frequencies of IL-17$^+$ effector/memory CD4$^+$ T cells are comparable. This reduction in Th1 effector/memory cells results from impaired persistence rather than from an impaired induction of Th1 effector/memory cells. When polarized into Th1 cells *in vitro*, Hopx$^{-/-}$ Th1-cell differentiation into IFN-γ expressing cells was not impaired. Polarized Th1 cells with knocked down *Hopx* expression did not persist in their hosts upon adoptive transfer. They also could not be rescued by antigenic stimulation. Th1 cells with knocked down *Hopx* expression failed to induce transfer colitis in Rag1$^{-/-}$ mice, as compared with control Th1 cells. Th1 cells with knocked down Hopx expression did not persist in spleen, lymph nodes, or colon, and chronic inflammation did not develop. In a model of OVA-induced arthritis, Hopx-deficient, OVA-specific Th1 cells induced only mild inflammation, as compared with WT Th1 cells. Interestingly, in this model, the Hopx-deficient Th1 cells persisted in the spleen and lymph nodes, but not in the inflamed joint. *In vitro*, Hopx-deficient Th1 cells show an increased susceptibility to Fas-mediated apoptosis. The observation that in the OVA-induced arthritis model, Hopx-deficient Th1 cells persist in spleen and lymph nodes, but not in the joint, the only place where the cationized antigen is displayed, argues that also *in vivo* Fas-mediated apoptosis may be involved in the elimination of Hopx-deficient Th1 cells. Th1 cells have been described to be more susceptible to Fas-mediated apoptosis than Th2 [14–17] and Th17 cells [18–20]. However, Th1 cells can be long lived and drive chronic inflammation [6–13], and can persist as effector/memory cells efficiently [24–26, 65]. Obviously, compensatory regulatory mechanisms have to exist in those cells. *Hopx* qualifies as such a compensatory gene, antagonizing Fas-mediated apoptosis of Th1 cells and promoting their persistence. Impaired T-cell apoptosis has been shown for mucosal Th1 cells in Crohn's disease, and associated with the sustained inflammatory response [66, 67]. The description of Hopx as a novel regulator of T-cell apoptosis will improve understanding mechanisms underlying systemic autoimmunity and offer possibilities for the development of new therapeutic approaches. Interestingly, also other lymphoid immune cells including B, CD8 and NK cells apparently do express Hopx (http://genome.ucsc.edu/cgi-bin/hgGateway). In summary, the description of Hopx as an anti-apoptotic factor, critical for the survival of repeatedly reactivated Th1 effector/memory cells and their ability to induce and maintain chronic inflammation, defines a new function of Hopx, and raises the question whether this function is also relevant for other immune cell types.

Materials and methods

Mice and reagents

BALB/c, C57BL/6, Rag-1$^{-/-}$, and OVA-TCR$^{tg/tg}$ DO11.10 or OT2 mice were purchased from The Jackson Laboratory or Charles River Laboratories and were bred under specific pathogen-free conditions in our animal facility. T-bet-deficient mice were a kind gift from J. Penninger, Vienna, Austria. Hopx-deficient mice were obtained from Jonathan Epstein and genotyped by using following primers (Hopx$^{-/-}$ and Hopx wt fw Primer: 5′-GCAGCACTT-GAGGCGCTTCCTCAGTATAC-3′, Hopx wt rv Primer: 5′-CCTT GTTGAAGTTGTACTCCAGGAT-3′, Hopx$^{-/-}$ rv Primer: 5′-CACGG CTTACGGCAATAATGCCTTT-3′). Hopx-deficient mice were backcrossed onto the C57BL/6 background for at least six generations. In the *in vitro* experiments and the *in vivo* animal experiments, Hopx-deficient cells and animals, respectively, were compared with their heterozygous and WT littermates.

All animal experiments were performed in accordance with institutional, state, and federal guidelines (Landesamt für Gesundheit und Soziales (LAGeSo), Berlin, Germany). Reagents were purchased from Sigma unless stated otherwise.

Isolation of human lymphocytes

PBMC from buffy coats from healthy donors were isolated by density gradient centrifugation (Lymphocyte separation medium, PAA) followed by fluorescence-activated cell sorting (see below).

Flow cytometry

The following antibodies directed against murine antigens were either purified from hybridoma supernatants and conjugated in-house or purchased as indicated: anti-CD3 (145-2C11), anti-CD4 (GK1.5), anti-CD44 (IM7), anti-CD62L (MEL14), anti-Vβ5.1,5.2 TCR (MR9-4, BD Pharmingen), anti-IL-4 (11B11, BD Pharmingen), anti-IFN-γ (AN18.17.24), anti-TNF-α (MP6-XT22, Caltag),

anti-IL-17 (TC11-18H10.1, BioLegend). Antibodies recognizing human antigens were obtained from BD Pharmingen unless stated otherwise: anti-CD3 (OKT3, in-house conjugate), anti-CD4 (TT1, in-house conjugate), anti-CD27 (L128), anti-CD45RA (HI100), anti-CRTh2 (BM16), anti-CCR5 (2D7/CCR5), anti-CD25 (2A3), anti-CD127 (R34.34, Immunotech). Cells were counted by using FlowCount Beads (Beckman Coulter). Flow cytometric analysis of apoptosis was performed using the Annexin-V-FLUOS staining kit (BD Biosciences). Data were collected with CellQuest (BD Biosciences) and FCS Express (De Novo) software on a FACSCalibur (BD Biosciences). Cells were separated by fluorescence-activated cell sorting (FACSAria, FACSDiVa, BD Biosciences).

Cell culture and apoptosis induction

Naïve $CD4^+CD62L^+$ lymphocytes from 6 to 8-wk-old DO11.10 or OT2 mice were isolated and polarized under Th1 or Th2 conditions as described elsewhere [29]. Irradiated (30Gy) congenic BALB/c or C57BL/6 splenocytes were used as APC at a ratio of 5:1 and the cognate peptide $ova_{323–339}$ (R. Volkmer-Engert, Humboldt University of Berlin, Germany) was added at 0.5 μM. Alternatively, plates were coated with 3 μg/mL anti-CD3 (145-2C11, BD Biosciences) in PBS, and Th cells were plated at a density of 2×10^6 cells/mL in medium plus 1 μg/mL soluble anti-CD28 (37.51). For Th17 differentiation, cells were stimulated in the presence of 1 ng/mL TGF-β (R&D Systems), 20 ng/mL IL-6 (R&D Systems), and 20 ng/mL IL-23 (R&D Systems) as well as 10 μg/mL anti-IL-4 (11B11) and 10 μg/mL anti-IFN-γ (AN18.17.24). Every 6 days, viable Th cells were harvested and restimulated under the original conditions, except that 10 ng/mL murine IL-2 (R&D Systems) was added to the Th1 and Th2 cultures.

To induce apoptosis, viable repeatedly restimulated Th cells were incubated with 2 μg/mL agonistic anti-Fas antibody (Jo2, BD PharMingen).

Mitogenic restimulation and intracellular cytokine staining

Cells were restimulated with 10 ng/mL PMA and 1 μg/mL ionomycin. For intracellular staining of cytokines, T cells were stimulated for 2 h with PMA/ionomycin and an additional 3 h with 5 μg/mL of brefeldin A. Cells were fixed with 2% formaldehyde in PBS for 15 min at room temperature and permeabilized with 0.5% w/v saponin.

mRNA quantification

Nucleospin RNAII (Macherey Nagel) was used for RNA isolation, TaqMan Reverse Transcription Reagents (Applied Biosystem) for cDNA synthesis, and LightCycler FastStart DNA Master SYBR Green I with a LightCycler 2.0 Instrument (Roche) for the PCR reaction. For normalization of murine and human cDNA, the transcripts for the housekeeping genes hypoxanthine guanine phosphoribosyl transferase and ubiquitin ligase H5 (UbcH5) were quantified, respectively. Relative expression was calculated as follows: $E_t^{\Delta Cp\ \text{target gene (reference}-\text{sample)}} / E_h^{\Delta Cp\ \text{housekeeping gene (reference}-\text{sample)}}$, where Cp represents the crossing point and E represents the reaction efficiency, determined by serial dilution of DNA. Primer sequences and PCR conditions are provided in Supporting Information Table 1.

Immunoblot

Immunoblot was performed with a polyclonal Hopx-specific antibody (FL-73, Santa Cruz, 1:500) and anti-tubulin-α (DM1A, Calbiochem) followed by incubation with horseradish peroxidase-coupled anti-rabbit and anti-mouse (Santa Cruz) secondary antibodies, respectively. Individual bands were visualized with enhanced chemiluminescence (Amersham Biosciences) and the Intelligent Dark Box System LAS-3000 (Fujifilm).

Retroviral expression vectors and retroviral infection

The shRNA expression vector was generated as described previously [29]. The *Hopx* target sequence was designed with Dharmacon siRNA Design tool (5′-GCAGATCTGTTACGGA-CTA-3′). A non-specific control sequence was used as control. For retroviral overexpression of T-bet, the vector GFP-RV [68] was used, kindly provided by K. M. Murphy (Howard Hughes Medical Institute, St. Louis, MO, USA). Murine *t-bet* (cDNA generated from Th1 cells) was amplified introducing *Bgl*II and *Xho*I-compatible restriction sites and ligated into the vector upstream of the internal ribosome entry site (IRES)-*gfp* cassette. Sequences of primers and oligonucleotides for shRNA expression are listed in Supporting Information Table 1. Retroviral stocks were obtained by calcium phosphate co-transfection of HEK293 cells with the retrovirus packaging plasmids pECO and pCGP. The medium was replaced after 4 h, and viral supernatants were collected 24–48 h later. Th cells were infected 40 h after activation by 60 min centrifugation at $700 \times g$ at 30°C with viral supernatant and 8 μg/mL polybrene followed by replacement of the viral supernatant with the former culture supernatant.

Antigen-induced arthritis

Th1 polarized cells were generated *in vitro* and repeatedly activated for two cycles. On day 12 after the initial activation, viable resting Th1 cells were harvested and adoptively transferred i.v. into $Rag1^{-/-}$ mice. One day later, arthritis was induced by injection of 60 μg cationized OVA into one knee joint. The contralateral knee joint was left untreated. In total, 14 days

later, mice were sacrificed and knee joints were fixed in 10% formaldehyde, decalcified in saturated EDTA solution and embedded in paraffin. Knee joint sections were stained with hematoxylin/eosin and scored for exudates, granulocyte infiltration, hyperplasia, fibroblast proliferation/mononuclear cell infiltration, periarticular mononuclear cell infiltration (each scoring 0–3), bone/cartilage destruction (scoring 0–4), and an additional score of 1 for visible fibrin deposition and periarticular granulocyte infiltration as described elsewhere [29].

Immunohistochemistry

After decalcification, formalin-fixed paraffin-embedded tissue was subjected to a heat-induced epitope retrieval step before incubation with anti-CD3 (♯N1580, Dako, dilution 1:10) followed by biotinylated donkey anti-rabbit secondary antibody (Dianova) and the streptavidinAP kit (K5005, Dako). Nuclei were counter-stained with hematoxylin. Negative controls were performed omitting primary antibodies. Images were acquired using an AxioImager Z1 microscope equipped with a CCD camera (AxioCam MRm) and processed with Axiovision software (Carl Zeiss MicroImaging, Jena).

Transfer colitis

Naïve $CD4^+CD62L^+$ T cells isolated from C57BL/6 mice were activated with plate-bound anti-CD3 and soluble anti-CD28 under Th1 polarizing conditions (5 ng/mL IL-12, anti-IL-4). On day 2 after T-cell activation, retroviral infection was performed leading to stable expression of a Hopx targeting or a control shRNA and the viral reporter gene *gfp*. On day 6, GFP^+ cells were isolated by FACS and i.v. injected into $Rag1^{-/-}$ recipient mice. Following the Th-cell reconstitution, mice were weighted daily and inspected for clinical signs of disease. Experiments typically lasted between 8 and 10 wk. Mice were sacrificed, and colons were removed and fixed with 4% w/v formalin. Paraffin-embedded sections were cut and stained with hematoxylin and eosin. Inflammation was scored as described earlier [69]: grades 0–1, no colitis; grade 2, moderate colitis; and grades 3–4 severe colitis.

Microarray experiments

Microarray experiments and analysis were performed as described elsewhere [29]. For the previous gene expression profiling of once and repeatedly activated Th1 cells, Murine Genome U74A version 2 GeneChip arrays (Affymetrix) were used [29]. The comparison of the gene expression in TCR transgenic WT Th1 cells cultured for 12 days with $Hopx^{+/-}$ and $Hopx^{-/-}$ Th1 cells was performed using Murine Genome 430A version 2 GeneChip arrays (Affymetrix).

All microarray data are publicly available in GEO. The results for once *versus* repeatedly activated Th1 cells can be found under the GEO accession number GSE15733 and significant genes are available in Bioretis (http://www.bioretis-analysis.de) after login with number SPL_294 as described in GEO. The results for $Hopx^{-/-}$ *versus* Hopx-expressing Th1 cells can be found under the GEO accession number GSE24437.

Statistical

Statistical significance was calculated using the two-tailed Mann–Whitney test ($^*p<0.05$; $^{**}p<0.01$; and $^{***}p<0.005$).

Acknowledgements: The authors thank Simone Spieckermann and Katja Grollich as well as Gabriele Fernahl for excellent technical assistance in immunohistochemistry experiments, Toralf Kaiser, Jenny Kirsch and Katharina Raba for excellent support with the cell sorting experiments. The authors also thank D. Trono (Ecole Polytechnique Fédérale de Lausanne, Switzerland) for provision of the pLVTHM vector; L. Van Parijs (Massachusetts Institute of Technology, Cambridge, Massachusetts) for pLL3.7; and K. M. Murphy (Howard Hughes Medical Institute, St. Louis, Missouri) for GFP-RV. This work was supported by the German Ministry of Education and Research (BMBF grant 1KW9913 and 0313068), within the National Genome Research Network (NGFN 1–2, BMBF grant 01GS0110, 01GS0160, 01GS0413), by the German Science Foundation (SFB 650, SFB 633), and by the European Commission (MUGEN, AUTOCURE). U. N. was recipient of a Boehringer Ingelheim Fond stipend, K. T. received a Humboldt Foundation stipend, I. A. and M. H. L. received stipends of Miltenyi Biotech and contributions of I. L. were made possible by DFG funding through the Berlin-Brandenburg School for Regenerative Therapies GSC 203.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

References

1 **Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. and Coffman, R. L.,** Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* 1986. **136**: 2348–2357.

2 **Jacobson, N. G., Szabo, S. J., Weber-Nordt, R. M., Zhong, Z., Schreiber, R. D., Darnell, J. E., Jr. and Murphy, K. M.,** Interleukin 12 signaling in T helper type 1 (Th1) cells involves tyrosine phosphorylation of signal transducer and activator of transcription (Stat)3 and Stat4. *J. Exp. Med.* 1995. **181**: 1755–1762.

3 **Szabo, S. J., Kim, S. T., Costa, G. L., Zhang, X., Fathman, C. G. and Glimcher, L. H.,** A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 2000. **100**: 655–669.

4 **Mullen, A. C., High, F. A., Hutchins, A. S., Lee, H. W., Villarino, A. V., Livingston, D. M., Kung, A. L. *et al.*,** Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. *Science* 2001. **292**: 1907–1910.

5 **Szabo, S. J., Sullivan, B. M., Stemmann, C., Satoskar, A. R., Sleckman, B. P. and Glimcher, L. H.,** Distinct effects of T-bet in TH1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells. *Science* 2002. **295**: 338–342.

6 **Powrie, F., Leach, M. W., Mauze, S., Menon, S., Caddle, L. B. and Coffman, R. L.,** Inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RBhi CD4+ T cells. *Immunity* 1994. **1**: 553–562.

7 **Ito, H. and Fathman, C. G.,** CD45RBhigh CD4+ T cells from IFN-gamma knockout mice do not induce wasting disease. *J. Autoimmun.* 1997. **10**: 455–459.

8 **Iqbal, N., Oliver, J. R., Wagner, F. H., Lazenby, A. S., Elson, C. O. and Weaver, C. T.,** T helper 1 and T helper 2 cells are pathogenic in an antigen-specific model of colitis. *J. Exp. Med.* 2002. **195**: 71–84.

9 **Neurath, M. F., Weigmann, B., Finotto, S., Glickman, J., Nieuwenhuis, E., Iijima, H., Mizoguchi, A. *et al.*,** The transcription factor T-bet regulates mucosal T cell activation in experimental colitis and Crohn's disease. *J. Exp. Med.* 2002. **195**: 1129–1143.

10 **Luger, D., Silver, P. B., Tang, J., Cua, D., Chen, Z., Iwakura, Y., Bowman, E. P. *et al.*,** Either a Th17 or a Th1 effector response can drive autoimmunity: conditions of disease induction affect dominant effector category. *J. Exp. Med.* 2008. **205**: 799–810.

11 **Bettelli, E., Sullivan, B., Szabo, S. J., Sobel, R. A., Glimcher, L. H. and Kuchroo, V. K.,** Loss of T-bet, but not STAT1, prevents the development of experimental autoimmune encephalomyelitis. *J. Exp. Med.* 2004. **200**: 79–87.

12 **Kroenke, M. A., Carlson, T. J., Andjelkovic, A. V. and Segal, B. M.,** IL-12- and IL-23-modulated T cells induce distinct types of EAE based on histology, CNS chemokine profile, and response to cytokine inhibition. *J. Exp. Med.* 2008. **205**: 1535–1541.

13 **Maffia, P., Brewer, J. M., Gracie, J. A., Ianaro, A., Leung, B. P., Mitchell, P. J., Smith, K. M. *et al.*,** Inducing experimental arthritis and breaking self-tolerance to joint-specific antigens with trackable, ovalbumin-specific T cells. *J. Immunol.* 2004. **173**: 151–156.

14 **Zhang, X., Brunner, T., Carter, L., Dutton, R. W., Rogers, P., Bradley, L., Sato, T. *et al.*,** Unequal death in T helper cell (Th)1 and Th2 effectors: Th1, but not Th2, effectors undergo rapid Fas/FasL-mediated apoptosis. *J. Exp. Med.* 1997. **185**: 1837–1849.

15 **Varadhachary, A. S., Perdow, S. N., Hu, C., Ramanarayanan, M. and Salgame, P.,** Differential ability of T cell subsets to undergo activation-induced cell death. *Proc. Natl. Acad. Sci. USA* 1997. **94**: 5778–5783.

16 **Ramsdell, F., Seaman, M. S., Miller, R. E., Picha, K. S., Kennedy, M. K. and Lynch, D. H.,** Differential ability of Th1 and Th2 T cells to express Fas ligand and to undergo activation-induced cell death. *Int. Immunol.* 1994. **6**: 1545–1553.

17 **Sato, T., Irie, S., Kitada, S. and Reed, J. C.,** FAP-1: a protein tyrosine phosphatase that associates with Fas. *Science* 1995. **268**: 411–415.

18 **Yu, Y., Iclozan, C., Yamazaki, T., Yang, X., Anasetti, C., Dong, C. and Yu, X. Z.,** Abundant c-Fas-associated death domain-like interleukin-1-converting enzyme inhibitory protein expression determines resistance of T helper 17 cells to activation-induced cell death. *Blood* 2009. **114**: 1026–1028.

19 **Shi, G., Ramaswamy, M., Vistica, B. P., Cox, C. A., Tan, C., Wawrousek, E. F., Siegel, R. M. and Gery, I.,** Unlike Th1, Th17 cells mediate sustained autoimmune inflammation and are highly resistant to restimulation-induced cell death. *J. Immunol.* 2009. **183**: 7547–7556.

20 **Fang, Y., Yu, S., Ellis, J. S., Sharav, T. and Braley-Mullen, H.,** Comparison of sensitivity of Th1, Th2, and Th17 cells to Fas-mediated apoptosis. *J. Leukoc. Biol.* 2010. **87**: 1019–1028.

21 **Morita, Y., Yamamura, M., Kawashima, M., Harada, S., Tsuji, K., Shibuya, K., Maruyama, K. and Makino, H.,** Flow cytometric single-cell analysis of cytokine production by CD4+ T cells in synovial tissue and peripheral blood from patients with rheumatoid arthritis. *Arthritis Rheum.* 1998. **41**: 1669–1676.

22 **Fuss, I. J., Neurath, M., Boirivant, M., Klein, J. S., de la Motte, C., Strong, S. A., Fiocchi, C. and Strober, W.,** Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. *J. Immunol.* 1996. **157**: 1261–1270.

23 **Yamada, H., Nakashima, Y., Okazaki, K., Mawatari, T., Fukushi, J. I., Kaibara, N., Hori, A. *et al.*,** Th1 but not Th17 cells predominate in the joints of patients with rheumatoid arthritis. *Ann. Rheum. Dis.* 2008. **67**: 1299–1304.

24 **Swain, S. L.,** Generation and *in vivo* persistence of polarized Th1 and Th2 memory cells. *Immunity* 1994. **1**: 543–552.

25 **Lohning, M., Hegazy, A. N., Pinschewer, D. D., Busse, D., Lang, K. S., Hofer, T., Radbruch, A. *et al.*,** Long-lived virus-reactive memory T cells generated from purified cytokine-secreting T helper type 1 and type 2 effectors. *J. Exp. Med.* 2008. **205**: 53–61.

26 **Harrington, L. E., Janowski, K. M., Oliver, J. R., Zajac, A. J. and Weaver, C. T.,** Memory CD4 T cells emerge from effector T-cell progenitors. *Nature* 2008. **452**: 356–360.

27 **Chen, F., Kook, H., Milewski, R., Gitler, A. D., Lu, M. M., Li, J., Nazarian, R. *et al.*,** Hop is an unusual homeobox gene that modulates cardiac development. *Cell* 2002. **110**: 713–723.

28 **Shin, C. H., Liu, Z. P., Passier, R., Zhang, C. L., Wang, D. Z., Harris, T. M., Yamagishi, H. *et al.*,** Modulation of cardiac growth and development by HOP, an unusual homeodomain protein. *Cell* 2002. **110**: 725–735.

29 **Niesner, U., Albrecht, I., Janke, M., Doebis, C., Loddenkemper, C., Lexberg, M. H., Eulenburg, K. *et al.*,** Autoregulation of Th1-mediated inflammation by twist1. *J. Exp. Med.* 2008. **205**: 1889–1901.

30 **Sallusto, F., Lenig, D., Forster, R., Lipp, M. and Lanzavecchia, A.,** Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 1999. **401**: 708–712.

31 **De Jong, R., Brouwer, M., Hooibrink, B., Van der Pouw-Kraan, T., Miedema, F. and Van Lier, R. A.,** The CD27- subset of peripheral blood memory CD4+ lymphocytes contains functionally differentiated T lymphocytes that develop by persistent antigenic stimulation *in vivo*. *Eur. J. Immunol.* 1992. **22**: 993–999.

32 **Fritsch, R. D., Shen, X., Sims, G. P., Hathcock, K. S., Hodes, R. J. and Lipsky, P. E.,** Stepwise differentiation of CD4 memory T cells defined by expression of CCR7 and CD27. *J. Immunol.* 2005. **175**: 6489–6497.

33 **Bonecchi, R., Bianchi, G., Bordignon, P. P., D'Ambrosio, D., Lang, R., Borsatti, A., Sozzani, S. *et al.*,** Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J. Exp. Med.* 1998. **187**: 129–134.

34 **Liu, W., Putnam, A. L., Xu-Yu, Z., Szot, G. L., Lee, M. R., Zhu, S., Gottlieb, P. A. *et al.*,** CD127 expression inversely correlates with FoxP3 and suppressive function of human $CD4^+$ Treg cells. *J. Exp. Med.* 2006. **203**: 1701–1711.

35 **Nagata, K., Tanaka, K., Ogawa, K., Kemmotsu, K., Imai, T., Yoshie, O., Abe, H. *et al.*,** Selective expression of a novel surface molecule by human Th2 cells *in vivo*. *J. Immunol.* 1999. **162**: 1278–1286.

36 **Adayev, T., Estephan, R., Meserole, S., Mazza, B., Yurkow, E. J. and Banerjee, P.,** Externalization of phosphatidylserine may not be an early signal of apoptosis in neuronal cells, but only the phosphatidylserine-displaying apoptotic cells are phagocytosed by microglia. *J. Neurochem.* 1998. **71**: 1854–1864.

37 **Fadok, V. A., Voelker, D. R., Campbell, P. A., Cohen, J. J., Bratton, D. L. and Henson, P. M.,** Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J. Immunol.* 1992. **148**: 2207–2216.

38 **Verhoven, B., Krahling, S., Schlegel, R. A. and Williamson, P.,** Regulation of phosphatidylserine exposure and phagocytosis of apoptotic T lymphocytes. *Cell Death Differ.* 1999. **6**: 262–270.

39 **Zwaal, R. F. and Schroit, A. J.,** Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. *Blood* 1997. **89**: 1121–1132.

40 **Das, P., Estephan, R. and Banerjee, P.,** Apoptosis is associated with an inhibition of aminophospholipid translocase (APTL) in CNS-derived HN2-5 and HOG cells and phosphatidylserine is a recognition molecule in microglial uptake of the apoptotic HN2-5 cells. *Life Sci.* 2003. **72**: 2617–2627.

41 **Putz, U., Harwell, C. and Nedivi, E.,** Soluble CPG15 expressed during early development rescues cortical progenitors from apoptosis. *Nat. Neurosci.* 2005. **8**: 322–331.

42 **Wakabayashi, Y., Watanabe, H., Inoue, J., Takeda, N., Sakata, J., Mishima, Y., Hitomi, J. *et al.*,** Bcl11b is required for differentiation and survival of alphabeta T lymphocytes. *Nat. Immunol.* 2003. **4**: 533–539.

43 **Coffield, V. M., Helms, W. S., Jiang, Q. and Su, L.,** Galpha13 mediates a signal that is essential for proliferation and survival of thymocyte progenitors. *J. Exp. Med.* 2004. **200**: 1315–1324.

44 **Kurokawa, M., Zhao, C., Reya, T. and Kornbluth, S.,** Inhibition of apoptosome formation by suppression of Hsp90beta phosphorylation in tyrosine kinase-induced leukemias. *Mol. Cell Biol.* 2008. **28**: 5494–5506.

45 **Zhang, Y., Wang, J. S., Chen, L. L., Cheng, X. K., Heng, F. Y., Wu, N. H. and Shen, Y. F.,** Repression of hsp90beta gene by p53 in UV irradiation-induced apoptosis of Jurkat cells. *J. Biol. Chem.* 2004. **279**: 42545–42551.

46 **Li, N., Zheng, Y., Chen, W., Wang, C., Liu, X., He, W., Xu, H. and Cao, X.,** Adaptor protein LAPF recruits phosphorylated p53 to lysosomes and triggers lysosomal destabilization in apoptosis. *Cancer Res.* 2007. **67**: 11176–11185.

47 **Chen, W., Li, N., Chen, T., Han, Y., Li, C., Wang, Y., He, W. *et al.*,** The lysosome-associated apoptosis-inducing protein containing the pleckstrin homology (PH) and FYVE domains (LAPF), representative of a novel family of PH and FYVE domain-containing proteins, induces caspase-independent apoptosis *via* the lysosomal-mitochondrial pathway. *J. Biol. Chem.* 2005. **280**: 40985–40995.

48 **Pan, X., Zhao, J., Zhang, W. N., Li, H. Y., Mu, R., Zhou, T., Zhang, H. Y. *et al.*,** Induction of SOX4 by DNA damage is critical for p53 stabilization and function. *Proc. Natl. Acad. Sci. USA* 2009. **106**: 3788–3793.

49 **Morrissey, P. J., Charrier, K., Braddy, S., Liggitt, D. and Watson, J. D.,** CD4+ T cells that express high levels of CD45RB induce wasting disease when transferred into congenic severe combined immunodeficient mice. Disease development is prevented by cotransfer of purified CD4+ T cells. *J. Exp. Med.* 1993. **178**: 237–244.

50 **Kook, H. and Epstein, J. A.,** Hopping to the beat. Hop regulation of cardiac gene expression. *Trends Cardiovasc. Med.* 2003. **13**: 261–264.

51 **Kook, H., Lepore, J. J., Gitler, A. D., Lu, M. M., Wing-Man Yung, W., Mackay, J., Zhou, R. *et al.*,** Cardiac hypertrophy and histone deacetylase-dependent transcriptional repression mediated by the atypical homeodomain protein Hop. *J. Clin. Invest.* 2003. **112**: 863–871.

52 **Watanabe, K., Okamoto, K. and Yonehara, S.,** Sensitization of osteosarcoma cells to death receptor-mediated apoptosis by HDAC inhibitors through downregulation of cellular FLIP. *Cell Death Differ.* 2005. **12**: 10–18.

53 **Bonnotte, B., Favre, N., Reveneau, S., Micheau, O., Droin, N., Garrido, C., Fontana, A. *et al.*,** Cancer cell sensitization to fas-mediated apoptosis by sodium butyrate. *Cell Death Differ.* 1998. **5**: 480–487.

54 **Chopin, V., Toillon, R. A., Jouy, N. and Le Bourhis, X.,** Sodium butyrate induces P53-independent, Fas-mediated apoptosis in MCF-7 human breast cancer cells. *Br. J. Pharmacol.* 2002. **135**: 79–86.

55 **Kim, H. R., Kim, E. J., Yang, S. H., Jeong, E. T., Park, C., Lee, J. H., Youn, M. J. *et al.*,** Trichostatin A induces apoptosis in lung cancer cells *via* simultaneous activation of the death receptor-mediated and mitochondrial pathway?. *Exp. Mol. Med.* 2006. **38**: 616–624.

56 **Carew, J. S., Giles, F. J. and Nawrocki, S. T.,** Histone deacetylase inhibitors: mechanisms of cell death and promise in combination cancer therapy. *Cancer Lett.* 2008. **269**: 7–17.

57 **Bolden, J. E., Peart, M. J. and Johnstone, R. W.,** Anticancer activities of histone deacetylase inhibitors. *Nat. Rev. Drug. Discov.* 2006. **5**: 769–784.

58 **Huang, L.,** Targeting histone deacetylases for the treatment of cancer and inflammatory diseases. *J. Cell Physiol.* 2006. **209**: 611–616.

59 **Grabiec, A. M., Tak, P. P. and Reedquist, K. A.,** Targeting histone deacetylase activity in rheumatoid arthritis and asthma as prototypes of inflammatory disease: should we keep our HATs on?. *Arthritis Res. Ther.* 2008. **10**: 226.

60 **Kee, H. J., Kim, J. R., Nam, K. I., Park, H. Y., Shin, S., Kim, J. C., Shimono, Y. *et al.*,** Enhancer of polycomb1, a novel homeodomain only protein-binding partner, induces skeletal muscle differentiation. *J. Biol. Chem.* 2007. **282**: 7700–7709.

61 **Schulz, E. G., Mariani, L., Radbruch, A. and Hofer, T.,** Sequential polarization and imprinting of type 1 T helper lymphocytes by interferon-gamma and interleukin-12. *Immunity* 2009. **30**: 673–683.

62 **Harvey, R. P., Lai, D., Elliott, D., Biben, C., Solloway, M., Prall, O., Stennard, F. *et al.*,** Homeodomain factor Nkx2-5 in heart development and disease. *Cold Spring Harb. Symp. Quant. Biol.* 2002. **67**: 107–114.

63 **Lyons, I., Parsons, L. M., Hartley, L., Li, R., Andrews, J. E., Robb, L. and Harvey, R. P.,** Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeo box gene Nkx2-5. *Genes. Dev.* 1995. **9**: 1654–1666.

64 **Stennard, F. A., Costa, M. W., Elliott, D. A., Rankin, S., Haast, S. J., Lai, D., McDonald, L. P. *et al.*,** Cardiac T-box factor Tbx20 directly interacts with Nkx2-5, GATA4, and GATA5 in regulation of gene expression in the developing heart. *Dev. Biol.* 2003. **262**: 206–224.

65 **Tokoyoda, K., Zehentmeier, S., Hegazy, A. N., Albrecht, I., Grun, J. R., Lohning, M. and Radbruch, A.,** Professional memory CD4+ T lymphocytes preferentially reside and rest in the bone marrow. *Immunity* 2009. **30**: 721–730.

66 **Boirivant, M., Marini, M., Di Felice, G., Pronio, A. M., Montesani, C., Tersigni, R. and Strober, W.,** Lamina propria T cells in Crohn's disease and other gastrointestinal inflammation show defective CD2 pathway-induced apoptosis. *Gastroenterology* 1999. **116**: 557–565.

67 **Ina, K., Itoh, J., Fukushima, K., Kusugami, K., Yamaguchi, T., Kyokane, K., Imada, A. *et al.*,** Resistance of Crohn's disease T cells to multiple apoptotic signals is associated with a Bcl-2/Bax mucosal imbalance. *J. Immunol.* 1999. **163**: 1081–1090.

68 **Afkarian, M., Sedy, J. R., Yang, J., Jacobson, N. G., Cereb, N., Yang, S. Y., Murphy, T. L. and Murphy, K. M.,** T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells. *Nat. Immunol.* 2002. **3**: 549–557.

69 **Asseman, C., Read, S. and Powrie, F.,** Colitogenic Th1 cells are present in the antigen-experienced T cell pool in normal mice: control by CD4+ regulatory T cells and IL-10. *J. Immunol.* 2003. **171**: 971–978.

Abbreviations: **CM:** central memory · **CRTh2:** chemo-attractant receptor-homologous molecule expressed on Th2 cells · **EM:** effector memory · **HOPX:** *homebox only protein* · **shRNA:** small hairpin RNA · **SRF:** serum-response factor

Full correspondence: Prof. Andreas Radbruch, German Rheumatism Research Center Berlin, Chariteplatz 1, 10117 Berlin, Germany
Fax: +49-30-28460-603
e-mail: radbruch@drfz.de

Current addresses: Christoph Loddenkemper, Institute of Pathology, Technische Universität München, Munich, Germany
Uwe Niesner, Abbott Products GmbH, Hannover, Germany

Received: 10/8/2010
Revised: 16/9/2010
Accepted: 20/9/2010
Accepted article online: 30/9/2010

3.8 Th-Zellen behalten ihre entzündungsfördernden Eigenschaften in Abwesenheit von T-bet

Zimmermann J, Kühl AA, Weber M, Grün JR, Löffler J, Haftmann C, Riedel R, Maschmeyer P, Lehmann K, Westendorf K, Mashreghi MF, Löhning M, Mack M, Radbruch A*, **Chang HD***. 2016. T-bet expression by Th cells promotes type 1 inflammation but is dispensable for colitis. *Mucosal Immunol*. 9(6):1487-1499. *doi: 10.1038/mi.2016.5*

Die Expression von T-bet in Th-Zellen ist assoziiert mit Pathogenität der Th-Zellen in chronischen Entzündungen. In dieser Studie haben wir untersucht wie sich T-bet-defiziente Th-Zellen sich im Modell der T-Zelltransfer-Kolitis anpassen. Wir konnten zeigen, dass Th-Zellen auch in Abwesenheit von T-bet eine Darmentzündung mit leicht veränderten histopathologischen Eigenschaften auslösen. Ohne T-bet differenzierten Th-Zellen in der Transferkolitis zu IL-17$^+$RORγt$^+$ Th17-Zellen. Interessanterweise behielten Th-Zellen die Kapazität IFN-γ zu exprimieren, was die Rolle von T-bet als Mastertranskriptionsfaktor für die IFN-γ-Expression in Frage stellt. Ohne T-bet sind Th-Zellen in ihrem Überleben beeinträchtigt und zeigen ein verändertes Migrationsverhältnis, sie wandern vermehrt in den Dünndarm. Des Weiteren wird durch T-bet-defiziente Th-Zellen die Polarisierung von klassisch-aktivierten, pro-inflammatorischen Typ 1 Monozyten und Makrophagen zu alternativ-aktivierten Typ 2 Monozyten und Makrophagen verändert.

Eine nähere Untersuchung des zellulären Infiltrats im entzündeten Darm gab Aufschluss darüber, dass in Abwesenheit von T-bet vermehrt eosinophile und neutrophile Granulozyten, jedoch weniger Monozyten und Makrophagen rekrutiert wurden. Die Veränderung des Infiltrats liegt dem veränderten Expressionsprofil für Chemokine der Th-Zellen zugrunde. In Abwesenheit von T-bet wird die Expression von Cxcl3 und Cxcl5 erhöht, beides Chemokine, die für die Rekrutierung von neutrophilen Granulozyten bekannt sind. Gleichzeitig wird die Expression von Ccl3, Ccl4 und Ccl5 reduziert, deren Hauptfunktion es ist CCR1- und CCR5-positive Monozyten und Makrophagen zu rekrutieren. Die Tatsache, dass der Transfer von IFN-γ-defizienten Th-Zellen keine Auswirkung auf die Darmentzündung hatte, zeigt, dass die Th-Zellen in diesem Fall ihre entzündungsfördernde Funktion hauptsächlich über die Rekrutierung von inflammatorischen Zellen, wie den Monozyten und Makrophagen bzw. den eosinophilen und neutrophilen Granulozyten ausüben.

Diese Studie hat gezeigt, dass Th-Zellen über verschiedene Mechanismen eine Entzündung auslösen können, die am Ende in ähnlichen klinischen Symptomen resultiert und deutet auf eine hohe Flexibilität des Immunsystems hin, chronische Entzündungen zu induzieren und aufrechtzuerhalten.

4 Diskussion

Th-Zellen spielen eine zentrale Rolle bei der Regulation einer Immunantwort. Sie können durch die Sekretion von Zytokinen und Chemokinen den Verlauf und die Art einer Immunreaktion steuern. Welche Zytokine und Chemokine die aktivierte Th-Zelle exprimiert hängt von den Signalen ab, die sie von Zellen des angeborenen Immunsystem bekommen. Diese instruktiven Signale vom angeborenen Immunsystem sind in der Regel Zytokine und kostimulatorische Liganden, die antigen-präsentierende Zellen aufgrund der Beschaffenheit des jeweiligen Krankheitserreger exprimieren. Die instruktiven Signale initiieren Differenzierungsprogramme in den Th-Zellen, die dazu führen, dass die aktivierten Th-Zellen ihre Effektorfunktion ausführen. Ist die Immunantwort erfolgreich im Sinne, dass der Krankheitserreger effektiv bekämpft wurde, stellt die Prägung des Differenzierungsprogramms bei einem erneuten Kontakt mit dem gleichen Krankheitserreger sicher, dass das Immunsystem mit den gleichen Effektormechanismen reagieren kann, auch ohne erneute instruktive Signale, es hat ein Gedächtnis. Die Prägung der jeweiligen Th-Zellprogramme ist stabil, sodass die Gedächtnisantwort auch in Gegenwart adverser Signale relativ robust bleibt. Gleichzeitig muss aber eine gewisse Flexibilität erhalten bleiben, die dem Immunsystem erlaubt regulatorisch einzugreifen, d.h. Effektorfunktionen oder die Zellen selbst intrinsisch oder über extrinische Signale zu modulieren, um z.B. exzessive Immunpathologie zu verhindern. Bei chronisch-entzündlichen Krankheiten, wie z.B. Autoimmunität, scheinen diese regulatorischen Mechanismen nicht mehr zu greifen.

Wir haben untersucht, wie aktivierte Th-Zellen für entzündungsfördernde und entzündungshemmende Funktionen geprägt sind und welche extrinsischen Signale die Funktion von entzündungsfördernden Th-Zellen modulieren können. Weiterhin haben wir untersucht, wie Th-Zellen auf chronische Aktivierung mit Antigen, wie sie in einer chronischen Entzündung passiert, reagieren, um ihre Funktion aufrechtzuerhalten. Sofern diese Anpassungen spezifisch für Th-Zellen sind, die an einer chronischen Entzündung beteiligt sind, und nicht für protektive Gedächtnis-Th-Zellen, könnten sie sich als vielversprechende Ziele für selektive Therapieansätze bei chronisch-entzündlichen Krankheiten anbieten.

4.1 Die pro-inflammatorische Prägung von Th-Zellen

In Abhängigkeit von instruktiven Signalen, die Th-Zellen während ihrer Aktivierung ausgesetzt sind, üben sie unterschiedliche Effektorfunktionen aus. Die Effektorfunktionen werden über die Expression von Zytokinen und Chemokinen definiert, die sowohl auf die Th-Zellen selbst aber

auch auf andere Zellen des adaptiven und angeborenen Arm des Immunsystems wirken. Historisch gesehen, werden die Th-Zellen anhand ihrer Effektorfunktion in verschiedene Th-Zellsubpopulationen eingeteilt [99]. Die Identifizierung von molekularen Mechanismen, die die Differenzierung von Th-Zellsubtypen verstärken und das Potential in einen alternativen Th-Zellsubtypen zu differenzieren, haben dazu geführt, dass die verschiedenen Th-Zelltypen als distinkte, stabile Th-Zellpopulationen betrachtet werden.

Zum Beispiel unterdrückt das Th2-induzierende Signal IL-4 über STAT6-Aktivierung die Th1-Differenzierung [100]. Die Inhibition der Th1-Differenzierung über IL-4 geschieht wahrscheinlich über GATA-3, welches durch STAT6 induziert wird und direkt die Expression von STAT4 und dadurch indirekt die Expression von IL-12Rβ2 inhibiert [101]. T-bet wiederum interagiert direkt mit GATA-3 und blockiert die Bindung von GATA-3 an die DNA [102]. Desweiteren unterdrückt T-bet auch direkt die Expression vieler Th2-spezifischer Gene [49]. Globale Chromatin-Immunpräzipitations (ChIP)-studien haben gezeigt, dass T-bet und GATA-3 eine große Schnittmenge an Genen haben an die sie binden [103]. T-bet und GATA-3 binden z.B. beide an den *Il4*- und *Ifng*-Lokus, allerdings mit unterschiedlichen Konsequenzen. T-bet-Bindung an den *Ifng*-Promoter führt zur Aktivierung der *Ifng*-Expression während T-bet-Bindung an das *Il4*-Gen in einer epigenetischen Stilllegung der *Il4*-Expression resultiert [51]. Hier wurde ein dedizierte regulatorische Region im IL-4-Gen identifiziert, der IL-4 *Silencer*, deren Deletion zu IL-4 Expression in Th1-Zellen führt [104]. Umgekehrt wird in Abhängigkeit von GATA-3 und STAT6 in Th2-Zellen der *Ifng*-Lokus epigenetisch geschlossen und für die Transkription ruhig gestellt [105]. Andererseits führt die Gegenwart von T-bet teilweise auch zu einer Veränderung des Bindeverhaltens von GATA-3, sodass die Bindung von GATA-3 an seine Th2-spezifischen Zielgene in Th1-Zellen in geringerem Maße mittels Chromatinimmunpräzipitation nachgewiesen werden konnte [106]. Die epigenetische Prägung, sprich die epigenetische Öffnung des einen Zytokingens bzw. die Schließung des anderen Zytokingens nimmt mit fortschreitender Zellteilung des jeweiligen Th-Zelltyps zu. Bei Th1- und Th2-Zellen konnte gezeigt werden, dass der *Il4*-Lokus bzw. der *Ifng*-Lokus mit fortschreitender Differenzierung in das transkriptionell inaktive Heterochromatin verschoben werden [22], andererseits werden die aktiven Gene progressiv und über größere Bereiche mittels DNA-Demethylierung geöffnet [36, 38, 39]. Bei Th17-Zellen ist die funktionelle und epigenetische Prägung weniger klar, auch wenn sie als separater Th-Zelltyp betrachtet werden [24]. Obwohl epigenetische Modifikationen im IL-17-Lokus in Form von Histonmodifikationen und DNA-Demethylierung nachgewiesen wurden [52, 53, 107] ist diese doch in Gegenwart von adversen Signalen wie IL-12 reversibel [53]. Diese epigenetische Instabilität prägt sich auch in einer Instabilität des Th17-Phänotyps aus [108].

4.2 Plastizität von Th-Zellen

Trotz der Vielfalt an molekularen Mechanismen, der wechselseitige Inhibition der unterschiedlichen Th-Zell-Differenzierungwege, gibt es Th-Zellen, die Zytokine unterschiedlicher Th-Zellsubtypen, wie z.B. IFN-γ und IL-4 [109, 110] oder IL-17 und IFN-γ [111, 112] koexprimieren. Wie solche Hybridzellen entstehen und wie sie sich funktionell von den „reinen" Th-Zellsubtypen unterscheiden war lange unklar.

Hegazy et al. konnte zeigen, dass *in vitro* differenzierte transgene Th2-Zellen, die einen spezifischen T-Zellrezeptor für das Lymphozytäres-Choriomeningitis-Virus (LCMV) tragen, nach adoptivem Transfer in Wildtyp-Wirtsmäuse und LCMV-Infektion anfingen IFN-γ zu exprimineren [110]. LCMV ist ein starker Th1-induzierender Virus, dennoch behielten die Th2 Zellen weiterhin ihre IL-4-Expression. Diese Th2+1-Zellen koexprimierten auch die Transkriptionsfaktoren GATA-3 und T-bet und hielten die Koexpression auch nach erneutem adoptiven Transfer in nicht-infizierte Mäuse über mehrere Monate aufrecht. Diese Studie zeigte zum ersten Mal, dass die Th1- und Th2-Programme stabil innerhalb einer Th-Zelle koexistieren können. Für das Erlangen des Th1-Phänotyps wurde der Interferon-Signalweg essentiell. Wurde den Th2-Zellen durch das Ausschalten des *Tbx21*-Gens die Fähigkeit genommen Th1-Funktionen zu erlangen, waren die Mäuse nicht in der Lage das Virus erfolgreich zu bekämpfen und zeigten stark erhöhte Immunpathologie. Die Fähigkeit von Th1- und Th2-Zellen sich Funktionen des jeweils anderen Th-Zelltyps anzueignen scheint daher ein wichtiger Mechanismus zu sein, um eine ineffektive Th-Zellantwort, in diesem Fall die anti-virale Th2-Antwort, und die damit verbundene Immunpathologie zu verhindern [113]. Interessanterweise kommt es anscheinend nie zu einer kompletten Konvertierung von Th1- zu Th2-Zellen bzw. umgekehrt. Grund hierfür ist wahrscheinlich die stabile epigenetische Prägung der Expression von IFN-γ und IL-4, bzw. von T-bet und GATA-3. Wir konnten das für IFN-γ auf Ebene der DNA-Methylierung zeigen [114, 115]. Sowohl GATA-3 [44] als auch T-bet [49] können ihre eigene Expression induzieren, wodurch eine positive Feedbackschleife die Expression der Transkriptionsfaktoren stabilisiert [46]. In Th1-Zellen bzw. in Th-Zellen, die anhand ihrer IFN-γ-Expression isoliert wurden, sind phylogenetisch konservierte DNA Regionen, wie z.B. der Promoter, selektiv demethyliert [114, 115].

Im Gegensatz zu Th1- und Th2-Zellen wird der Th17-Phänotyp als relativ unstabiles Differenzierungsstadium angesehen [53, 108]. Mehrere Studien haben gezeigt, dass *in vitro* generierte Th17-Zellen ihren Phänotyp nicht stabil aufrechterhalten können. Der adoptive Transfer von Th17-Zellen, selbst wenn sie anhand ihrer IL-17-Expression identifiziert und aufgereinigt wurden, resultierte in der Konvertierung der IL-17-exprimierenden Th-Zellen in IFN-

γ-exprimierende Zellen [108, 116, 117] und führte zum Konzept, dass Th17-Zellen eine große Plastizität besitzen. Dieses Konzept wurde mit Studien untermauert, die gezeigt haben, dass im *Il17*-Lokus keine stabilen epigenetischen Modifikationen induziert werden [53]. Allerdings konnten wir zeigen, dass Th17-Zellen, die *in vivo* generiert wurden, d.h. die mittels des IL-17-Sekretionsassays anhand ihrer IL-17-Expression direkt ex vivo isoliert wurden, durchaus stabil sein können und refraktär gegenüber einer Umpolarisierung in Th1- oder Th2-Zellen sind [118]. Diese Ergebnisse implizieren, dass es neben den Th17-instruierenden Signalen, die für die *in vitro* Differenzierung von Th17-Zellen genutzt werden, anscheinend bisher noch nicht identifizierte Signale gibt, die Th17-Zellen stabil prägen können. Die *in vitro* und *in vivo* generierten Th17-Zellen unterschieden sich in zwei zentralen Merkmalen: die Expression der IL12Rβ2-Kette und die stabile Prägung der IL-17-Expression. Die Th17-Zellen, die wir direkt *ex vivo* sortiert hatten, waren nicht in der Lage auf IL-12-Signale zu reagieren, da sie im Gegensatz zu den *in vitro* generierten Th17-Zellen keine IL12Rβ2-Kette exprimierten [111]. Die Stimulation der *ex vivo* isolierten Th17-Zellen mit IFN-γ, welches zur Induktion der Expression der IL12Rβ2-Kette führt [48, 111, 119], stellte die Reaktivität gegenüber IL-12 wieder her. Anders als die *in vitro* differenzierten Th17-Zellen behielten die *ex vivo* isolierten Th17-Zellen jedoch ihren Th17-Phänotyp, d.h. IL-17- und RORγt-Expression und erlangten zusätzlich Th1-Funktion, d.h. IFN-γ- und T-bet-Expression. Auch in menschlichen Th17-Zellklonen konnte gezeigt werden, dass durch IL-12-Aktivierung solche Th17+1-Zellen generiert werden können [94]. Beim Menschen konnte gezeigt werden, dass diese Th17+1-Zellen weiter zu sogenannten ex-Th17- oder nicht-klassischen Th1-Zellen differenzieren können, die sich von konventionellen Th1-Zellen durch die Expression von CD161, CCR6, RORC und IL-17-Rezeptor E unterscheiden [120]. Den nicht-klassischen Th1-Zellen wird eine erhöhte Pathogenität zugeschrieben, da deren Anzahl insbesondere im entzündeten Gewebe mit der Krankheitsaktivität korreliert [121, 122]. Desweiteren behalten die nicht-klassischen Th1-Zellen ihre epigenetische Prägung des RORC- und IL17A-Gens [123]. Was dies allerdings für die Funktion der nicht-klassischen Th1-Zellen bedeutet ist unklar.

In Mausmodellen für Typ 1 Diabetes wurde gezeigt, dass Th17-Zellen *in vivo* zu Th1-Zellen „konvertieren“ wenn sie die Entzündung auslösen [117, 124]. Allerdings ist nicht klar, ob es sich in diesen Fällen um nicht-klassische Th1-Zellen handelt, da die adoptiv transferierten Th17-Zellen *in vitro* generiert wurden, die in Gegenwart von IL-12 *in vitro* zu „klassischen“ Th1-Zellen konvertieren ohne nachweisliche Eigenschaften ihres Th17-Programms zu behalten [118]. Um die Konversion der Th17-Zellen *in vivo* verfolgen zu können, wurden „*fate-map*“-Mäuse untersucht in denen Th-Zellen, die IL-17 produzieren genetisch markiert werden [125]. In diesen

Mäusen zeigte sich im Modell der experimentellen autoimmunen Enzephalomyelitis (EAE) [125] und in einem Darmentzündungsmodell [126], dass fast alle Th-Zellen, die IFN-γ exprimierten ehemals IL-17-exprimierende Th-Zellen, d.h. nicht-klassische Th1-Zellen, waren. Wie sich nicht-klassische Th1-Zellen von klassischen Th1-Zellen in ihrem Beitrag zur Entzündung unterscheiden ist nicht bekannt. Möglich wäre, dass durch die Differenzierung der Th17-Zellen zu Th1-Zellen die Persistenz der antigen-spezifischen Th-Zellen gesteigert wird [127] und dies ein wichtiger Schritt zur Aufrechterhaltung von Entzündungen ist.

Vor kurzem wurde gezeigt, dass auch Th1-Zellen zu Th17-Zellen differenzieren können [128]. Th1-Zellen können demnach auch auf TGF-β und IL-6 reagieren und das Th17-Programm anschalten. Inwiefern diese „nicht-klassischen" Th17-Zellen und ihre Zwischenstufe - die Th1+17-Zellen - sich im Pathogenitätspotential von klassischen Th17-Zellen oder Th17+1-Zellen unterscheiden ist nicht klar.

Letztendlich könnte die Th-Zelle das funktionelle Repertoire beider pro-inflammatorischer Th-Zellen durch die Koexpression der beiden „Master"-Transkriptionsfaktoren T-bet und RORγt in sich vereinen. Die in meiner Gruppe erhobenen Transkriptomdaten von Th-Zellen die IFN-γ (Th1), IL-17 (Th17) oder beide Zytokine (Th17+1) exprimieren, haben gezeigt, dass Th17+1-Zellen viele Gene von Th1- und Th17-Zellen koexprimieren (Zimmermann et al., unveröffentlicht).

Die Expression von T-bet in Th-Zellen mit Th17-Phänotyp ist mit einer erhöhten Pathogenität der Th-Zellen assoziiert. Die Zytokine IL-6, IL-23 und IL-1β, waren in der Lage in Abwesenheit von TGF-β, die Th17-Differenzierung zu induzieren. Myelin-oligodendrozyten-glykoprotein-spezifische Th17-Zellen, die so generiert wurden koexprimierten RORγt und T-bet und lösen nach adoptiven Transfer in Rag-defiziente Mäuse eine Entzündung im zentralen Nervensystem aus, während Zellen die in Gegenwart von TGF-β1 ohne IL-23 dies nicht tun. Auch im EAE-Modell werden solche RORγt^{+}T-bet^{+} Th-Zellen direkt im entzündetem Gewebe detektiert [129]. In späteren Studien konnte gezeigt werden, dass TGF-β3 eine entscheidende Rolle in der Pathogenizität von Th17-Zellen spielt und ein Genexpressionsprofil induziert, welches sich fundamental von nicht-pathogenem TGF-β1-induzierten Th17-Zellen unterscheiden. Welche Veränderungen im Genexpressionsprofil letztendlich für die erhöhte Pathogenität verantwortlich sind, ist im Detail noch nicht geklärt. Diese Arbeit deutet allerdings auch darauf hin, dass TGF-β3 anscheinend unterhalb des Transkriptionsfaktor T-bet agiert, da eine exogene Gabe von TGF-β3 die Abwesenheit von T-bet für die Pathogenität der Th17-Zellen überwinden konnte [130].

Zusammenfassend zeigen, die bisherigen Erkenntnisse, dass Th-Zellen ein hohes Maß an Plastizität besitzen und nicht, wie vorher angenommen, dogmatisch in feste Kategorien, wie Th1, Th2, usw. eingruppiert werden können. Diese Plastizität erlaubt dem Immunsystem die Funktionen von Th-Zellen während einer Immunantwort abzustimmen, um z.B. Immunpathologie zu reduzieren, kann aber im Falle einer chronisch-entzündlichen Krankheit auch zu einer Steigerung der Immunpathologie führen.

4.3 T-bet als Regulator von Typ 1 Entzündung

Wie eben gerade beschrieben, ist T-bet-Expression ist mit einer erhöhten Pathogenität von Th-Zellen assoziiert. Es ist allerdings unklar welche Aspekte einer Entzündung T-bet kontrolliert, da in Abwesenheit von T-bet oft erst gar keine Entzündung entsteht. Frühere Studien haben gezeigt, dass T-bet absolut unerlässlich für die Manifestation einer Darmentzündung in der T-Zelltransferkolitis ist [91]. Der Transfer von T-bet-defizienten Th-Zellen in Rag-defiziente Mäuse am DRFZ zeigte jedoch, dass T-bet-defiziente Th-Zellen durchaus in der Lage sind eine Darmentzündung auszulösen [112]. Wir konnten mittlerweile zeigen, dass die widersprüchlichen Ergebnisse auf unterschiedliche Darmmikrobiota zurückzuführen sind (Zimmermann et al., unveröffentlicht).

In einer vergleichenden Analyse haben wir die Unterschiede zwischen einer Kolitis, die durch Wildtyp-Th-Zellen und die durch T-bet-defiziente Th-Zellen ausgelöst wird untersucht, um zu klären, welche Aspekte einer Entzündung, am Beispiel der chronischen Darmentzündung, durch T-bet reguliert werden. In der T-Zelltransferkolitis konnten wir zeigen, dass T-bet zum Überleben und der Lokalisierung von Th-Zellen im Kolon beiträgt. Das Überleben der Th-Zellen war mit einem erhöhten Verhältnis vom anti-apoptotischen Faktor Bcl-2 zum pro-apoptotischen Faktor Bim assoziiert. Ob dieses Bcl-2/Bim Verhältnis in Abhängigkeit von der T-bet-abhängigne microRNA-148a [131] verschoben wurde ist unklar. T-bet spielt auch eine wichtige Rolle bei der Unterdrückung der Th17-Differenzierung in vivo, da die Th-Zellen nach adoptivem Transfer fast ausschließlich zu RORγt^{+} IL-17-exprimierenden Th17-Zellen differenzierten [112]. Interessanterweise waren T-bet-defiziente Th-Zellen fast unbeeinträchtigt in ihrer Fähigkeit nach *in vitro* Restimulation IFN-γ zu exprimieren, was die Rolle von T-bet bei der Regulation der IFN-γ-Expression in Frage stellt. Tatsächlich gibt es Evidenz, dass IL-12/STAT4 allein ausreicht, um die IFN-γ-Expression in Abwesenheit von T-bet zu induzieren [49]. Ob IL-12 in unserem Fall zur IFN-γ-Expression beiträgt ist aber unklar. In Abwesenheit von T-bet führt die erhöhte Expression von RORγt und der Induktion des Th17-Programms zu einer Veränderung des Chemokinrezeptor- und Chemokin-Expressionsprofil. Dadurch wurde die Expression der

Chemokinrezeptoren CCR6 und CCR9 erhöht, deren Liganden CCL20 und CCL25 im Dünndarm exprimiert werden und die Th-Zellen dorthin rekrutieren [132, 133]. Gleichzeitig wurde die Expression von CXCR3 runterreguliert. Die Expression von CXCR3 ist T-bet-abhängig und reguliert die Migration in das entzündete Darmgewebe [134, 135]. Durch die Abwesenheit von T-bet wurde auch das Chemokinexpressionsprofil der Th-Zellen verändert. Als Konsequenz konnte auch ein verändertes Infiltrat an myeloiden Zellen im entzündeten Gewebe festgestellt werden. Während das zelluläre Infiltrat im entzündeten Gewebe bei Wildtyp-Th-Zellen von $CD64^+$ Monozyten und Makrophagen dominiert wird, waren in Gegenwart von T-bet-defizienten Th-Zellen eher eosinophile und neutrophile Granulozyten präsent [112]. In Abwesenheit von T-bet wurde auch die Polarisierung von Monozyten und Makrophagen des Typ 1 zu Typ 2 geändert. Typ 1 Monozyten und Makrophagen (M1) zeichnen sich durch die Expression von Sauerstoffradikalen und pro-inflammatorischen Zytokinen, wie IFN-γ, TNF-α, GM-CSF und IL-6 aus, während M2-Zellen eher anti-inflammatorische und Gewebe-reparierende Eigenschaften zugeschrieben wird [136]. Dennoch konnten wir zeigen, dass T-bet-defiziente Th-Zellen eine Darmentzündung auslösen, die sich histopathologisch und klinisch kaum von einer Darmentzündung unterscheidet, die von T-bet-exprimierenden Th Zellen induziert wird. Diese Ergebnisse zeigen deutlich, dass Entzündungen, wie die chronische Darmentzündung, unterschiedlichen Entzündungsmechanismen zugrunde liegen können, nicht nur in Abhängigkeit des genetischen Hintergrunds aber auch in Abhängigkeit der Mikrobiota, die manche Gene erst zum Tragen bringt. Dies bedeutet, dass auch bei Patienten mit chronisch-entzündlichen Darmerkrankungen, wie z.B. Morbus Crohn oder ulzerativer Kolitis, unterschiedliche T-Zelleffektormechanismen in Abhängigkeit der Mikrobiota zur Krankheit beitragen. Welche Bakterien von T-bet-defizienten und Wildtyp-Th-Zellen benötigt werden um eine Entzündung auszulösen und welche molekularen Mechanismen dem zugrunde liegen ist unklar. Auf jeden Fall scheinen die klinischen Beobachtungen diese Schlussfolgerung zu bestätigen, da das Ansprechen von Patienten mit chronisch-entzündlichen Darmerkrankungen auf Therapien, die auf bestimmte Effektormechanismen abzielen, wie z.B. TNF [137], IFN-γ [138], IL-23 [139] oder das Integrin VLA-4 [140], sehr heterogen ausfällt.

4.4 Konditionelle Expression des immunregulatorischen Zytokins IL-10

Wie schon erwähnt, ist ein wichtiger Aspekt der Plastizität von differenzierten Th-Zellen die Fähigkeit sich Funktionen anzueignen, um in einer Immunantwort die Immunpathologie in Grenzen zu halten [110, 113]. Eine entscheidende Funktion von Th-Zellen immunregulatorisch in eine Immunantwort einzugreifen ist die Expression des Zytokins IL-10. IL-10-Defizienz führt

zur spontanen Entwicklung einer Kolitis [64]. Später zeigte die selektive Deletion des *Il10*-Gens in T-Zellen, dass T-Zellen eine Hauptquelle für immunregulatorisches IL-10 sind [65]. In mehreren Entzündungsmodellen trägt IL-10, das von Th-Zellen exprimiert wird, zu einer erheblichen Reduktion der Immunpathologie bei [141-143]. Gleichzeitig kann IL-10 aber auch die Immunantwort gegen Pathogene einschränken, wie z.B. die Th1-vermittelte Immunantwort gegen Leishmania [144, 145] oder Toxoplasma [146].

Im Gegensatz zu Effektorzytokinen, wie IFN-γ, IL-4 und IL-17 ist IL-10 anscheinend nicht Teil des funktionellen Gedächtnis von Th-Zellen. Dies äußert sich zum einen dadurch, dass sowohl Maus als auch humane Th-Zellen ihre IL-10-Expression in nachfolgenden Aktivierungen nicht beibehalten, und dass das Il10-Gen epigenetisch nicht geprägt wird [66, 114]. Als wichtiges immunregulatorisches Zytokin kann IL-10 von allen Th-Zelltypen exprimiert werden. Allerdings sind die Signale, die unterschiedliche Th-Zelltypen für die Induktion der IL-10-Expression benötigen verschieden. Bei Th1-Zellen ist das instruktive und induzierende Signal für die IL-10 Expression das Zytokin IL-12 und sein Signalmolekül STAT4 [66]. Da IL-12 auch für die Differenzierung von Th-1 Zellen benötigt wird, haben wir postuliert, dass IL-12 in unterschiedlichen Phasen einer Immunantwort verschiedene Funktionen ausübt [147]. Während einer Primärantwort wirkt IL-12 pro-inflammatorisch, weil IL-12 für die stabile Induktion der IFN-γ-Expression benötigt wird [13]. Während einer Sekundärantwort könnte IL-12 jedoch durch seine IL-10-induzierende Aktivität anti-inflammatorisch wirken [66, 147]. Tatsächlich konnte gezeigt werden, dass IL-12 eine Entzündung über IL-10 unterdrücken kann [148]. In Th1-Zellen spielt auch das Notch-Signal eine wichtige Rolle, welches die Induktion von IL-10 durch STAT4-Aktivierung durch die Induktion von c-maf um ein vielfaches verstärkt [69, 149, 150].

In Th2-Zellen wird die IL-10-Expression durch IL-4 induziert [66]. In Th2-Zellen kommt es nach mehrmaliger Aktivierung in Gegenwart von IL-4 zu einer funktionellen Prägung der IL-10-Expression, d.h. IL-10 wird auch in Abwesenheit des instruktiven Signals IL-4 in nachfolgenden Aktivierungen exprimiert [31]. In Th2-Zellen wird die Prägung des *Il10*-Gens durch GATA-3 vermittelt [66, 68]. Warum sich eine stabile Prägung der IL-10-Expression erst nach mehreren Aktivierungszyklen in der Gegenwart von IL-4 ausprägt, obwohl GATA-3 schon früh in der Th2-Differenzierung aktiv ist [47, 151], ist unklar. Anscheinend braucht es noch weitere Faktoren, die erst nach mehrfacher Aktivierung induziert werden, um das *Il10*-Gen epigenetisch zu prägen. Ein möglicher Kandidat könnte der Transkriptionsfaktor Blimp-1 sein. Blimp-1 wird in Th-Zellen in chronischer Entzündung hochgeregelt [152] und ist ein wichtiger Faktor für die IL-10-Expression in Th-Zellen [150].

In Th17-Zellen ist die Koexpression von IL-10 auch mit einer immunregulatorischen Funktion assoziiert [142, 153]. Für die Induktion von IL-10 in Th17-Zellen spielt IL-1β eine wichtige Rolle [67]. Wir konnte allerdings auch zeigen, dass IL-1β die pro-inflammatorischen Eigenschaften von Th17-Zellen fördert [154]. IL-1β könnte ähnlich wie IL-12 bei Th1-Zellen also je nach Zeitpunkt und Kontext die pro- und anti-inflammatorischen Funktionen von Th17-Zellen regulieren.

4.5 Molekulare Adaptationen von Th-Zellen als Reaktion auf chronische Entzündung

Eine erfolgreiche Immunreaktion zeichnet sich dadurch aus, dass das Antigen innerhalb eines Zeitraums von mehreren Tagen bis Wochen komplett eliminiert wird. Der Großteil der beteiligten Th-Zellen stirbt in der sogenannten Kontraktionsphase bis auf wenige Zellen, die als Gedächtniszellen über längere Zeiträume erhalten bleiben [155]. Im Falle einer Autoreaktivität kann eine Th-Zelle ständig mit ihrem Antigen in Kontakt kommen ohne, dass das Antigen eliminiert werden kann. Wenn dann physiologische Regulations- und Toleranzmechanismen versagen kann es zu einer chronischen Entzündung und Autoimmunkrankheit kommen. Chronische Entzündungen scheinen sich in mehrfachen Aspekten von akuten Immunreaktionen zu unterscheiden. Diese Unterschiede spiegeln sich unter anderem in der Resistenz gegenüber einer regulatorischen bzw. therapeutischen Beeinflussung wieder. Während sich akute Immunreaktionen sehr effizient mittels immunsuppressiver Therapie unter Kontrolle bringen lassen, sprechen viele Patienten mit chronischen Entzündungen gar nicht erst auf immunsuppressive Therapien an [156] bzw. erleiden einen Rückfall sobald die Therapie gestoppt wird [157, 158]. Auch konnte gezeigt werden, dass Th-Zellen in chronischer Entzündung sich nicht mehr durch regulatorische T-Zellen (Treg) in ihrer Funktion unterdrücken lassen [159, 160]. Ein Mechanismus warum Th-Zellen in einer chronischen Entzündung nicht mehr von Treg kontrolliert werden, könnte über IL-2 gehen. Treg benötigen neben dem T-Zellrezeptor auch die Gegenwart von IL-2, um aktiviert zu werden [161, 162]. Da Treg selbst kein IL-2 exprimieren sind sie für ihre Aktivierung vom exogenen IL-2 der Effektor-T-Zellen abhängig. In Th1-Zellen wird die IL-2-Expression durch T-bet unterdrückt, indem T-bet mit der NF-κB-Komponente RelA interagiert und verhindert das RelA das *Il2*-Gen transaktivieren kann [163]. Im Lupus-Mausmodell ließ sich zeigen, dass ein Mangel an IL-2 zur Krankheitsentwicklung beiträgt [164]. Die Gabe von rekombinanten IL-2 führte im Mausmodell und bei Lupus-Patienten zu einer Aktivierung und Vermehrung der Treg und zu einer deutlichen Verbesserung der klinischen Symptome [164-166]

Basierend auf der Hypothese, dass sich insbesondere Effektor-Th-Zellen als zentrale Schaltstelle für Immunreaktionen im Verlauf einer chronischen Entzündung auf molekularer

Ebene verändern, haben wir untersucht, ob eine ständige, mehrfache Reaktivierung auch endogene Veränderungen in der Th-Zelle auslöst, die zur Chronifizierung der Entzündung beitragen. Um mögliche Veränderungen in den Th-Zellen auf globaler Ebene zu erfassen, haben wir Th-Zellen unter verschiedenen polarisierenden Bedingungen *in vitro* einfach und mehrfach restimuliert und deren Transkriptome verglichen. Die von uns identifizierten und näher untersuchten Gene *Twist1* und *Hopx* waren selektiv in mehrfach restimulierten Th1-Zellen hoch exprimiert [167, 168]. Für Twist1 konnte bereits gezeigt werden, dass Twist1 in Makrophagen die Aktivität von *nuclear factor 'kappa-light-chain-enhancer' of activated B-cells* (NF-κB) inhibiert und dadurch entzündungsfördernde Zytokine reduziert [169, 170]. Die Haploinsuffizienz von Twist1 bei gleichzeitiger Abwesenheit vom verwandten Twist2 führt zur systemischen Entzündung und Kachexie [169]. In Th-Zellen führte die ektopische Überexpression von Twist1 zu einer Reduktion der Expression der pro-inflammatorischen Zytokine IFN-γ und TNF-α [167]. Twist1 selbst wird durch STAT4, T-Zellrezeptorsignal und einem noch unbekanntem Signal von B-Zellen induziert und wird danach unabhängig von den induzierenden Signalen mit jeder Reaktivierung weiter hochgeregelt. [167]. In der frühen Th1-Entwicklung kann Twist1 mit Runx3 interagieren und so die Expression von IFN-γ unterdrücken [171]. Ob dieser Mechanismus aber auch bei mehrfach reaktivierten Th1-Zellen aktiv ist, ist unklar, da bei mehrfach aktivierten Th1-Zellen keine Beeinflussung der IFN-γ-Expression durch Twist1 zu beobachten war. Im Modell der ovalbumin-induzierten Arthritis führt der *knock-down* der Twist1-Expression durch retroviral-kodierte shRNA (*short-hairpin* RNA) zu einer Verstärkung der Entzündung im betroffenen Kniegelenk. Twist1 scheint daher dennoch eine wichtige Rolle bei einer Th1-zellintrinsischen negativen Rückkopplungsschleife, die zur Einschränkung der Th1-Funktion dient. Inwiefern die Twist1-vermittelten autoregulatorischen Mechanismen bei chronischer Entzündung greifen ist unklar, da gerade in Patienten mit schwerer Entzündung die Th-Zellen im entzündeten Gewebe hohe Mengen an Twist1 exprimieren [167]. Im Kontext einer chronischen Entzündung könnte Twist1 eher eine Rolle im Überleben der Th-Zellen spielen. Zusammen mit T-bet induziert Twist1 direkt die Expression der microRNA miR-148a. miR-148a bindet an die RNA von Bim und beeinflusst so die Bim-Bcl2-Balance zugunsten von Bcl2 [131]. Das Bim-Bcl2-Verhältnis ist entscheidend für das Überleben von Th-Zellen [172]. In Th-Zellen, die aus entzündetem Gewebe von Patienten mit chronischer Entzündung isoliert wurden, konnte eine Korrelation zwischen der Twist1- und miR-148a-Expression festgestellt werden [131].

Auch Hopx scheint das Überleben von Th1-Zellen zu fördern. Wird Hopx über shRNA-vermittelten knock-down inhibiert, können adoptiv transferierte Th1-Zellen nicht im Empfängertier überleben und lösen im T-Zelltransferkolitis-Modell keine Darmentzündung aus

[168]. Wie Hopx das Überleben der Th1-Zellen fördert ist noch nicht aufgeklärt. Hopx selbst hat keine eigene DNA-Bindeaktivität [173, 174] und muss über Interaktion mit anderen Proteinen seine Funktion ausüben. In Kardiomyozyten interagiert Hopx mit dem *serum response factor* (SRF) und reguliert die Proliferation und Differenzierung von Kardiomyozyten [173, 174]. In Th-Zellen konnten wir keine Interaktion zwischen Hopx und SRF nachweisen.

Zusammenfassend zeigen unsere Daten, dass mehrfach reaktivierte Th-Zellen molekulare Veränderungen durchlaufen, wie der Induktion der Expression von Twist1 und Hopx, die das Überleben der Th-Zellen im entzündeten Gewebe fördern und somit zu einer Chronifizierung der T-zellvermittelten Entzündung beitragen.

4.6 Selektives, therapeutisches Targeting von Immunzellen zur Behandlung von chronischen Entzündungen

Chronisch-entzündliche Krankheiten, wie Autoimmunkrankheiten, zeichnen sich dadurch aus, dass sie eine noch eine chronische Therapie benötigen. In den seltensten Fällen kann mittels konventioneller Immunsuppression eine therapie-freie Remission erreicht werden [157]. Obwohl durch moderne Therapien, die auf eine Unterdrückung des Immunsystems abzielen, in vielen Patienten ein Fortschreiten der Krankheit komplett zum Erliegen gebracht werden kann [175] führt ein Absetzen der Therapie oft zu einem Rückfall der Krankheit. Diese klinischen Beobachtungen deuten darauf hin, dass Faktoren die an einer chronischen Entzündung beteiligt sind, nicht durch konventionelle Immunsuppression eliminiert werden. Im Gegensatz dazu, zeigt die Therapie mittels Immunablation gefolgt von autologer Stammzelltransplantation (ASCT), dass die pathogene Komponente, die eine chronische Entzündung antreibt, eliminiert werden kann [176]. Bei dieser experimentellen Therapie wird in der Regel das gesamte Immunsystem mit Hilfe von anti-Thymozytenglobulin (ATG) depletiert und daraufhin mit autologen hämatopoietischen Stammzellen wieder rekonstituiert. In ca. 50% der Patienten wird dadurch eine therapie-freie Remission, sprich Heilung, erreicht [176, 177]. Warum die Immunablation zu einer Heilung führt und die Immunsuppression nicht ist noch nicht geklärt. Eine Hypothese ist, dass durch die Immunablation Zellen des immunologischen Gedächtnisses eliminiert werden, die durch die Immunsuppression nicht getroffen werden. Die Tatsache, dass trotz konventioneller Immunsuppression die T-Zellrezeptordiversität nicht beeinflusst wird [178] und auch Titer von Antikörpern, sowohl autoreaktive als auch protektive [179, 180], fast unverändert bleiben, deutet darauf hin, dass Zellen des immunologischen Gedächtnisses den Unterschied zwischen Immunsuppression und Immunablation ausmachen könnten. Bei der Immunablation mit ASCT kommt es zu einem Neustart des Immunsystems mit dem kompletten Verlust des immunologischen Gedächtnisses [177, 181]. Die Eliminierung des immunologischen

Gedächtnisses zusammen mit der vorübergehenden Immundefizienz bis ein neues Immunsystem regeneriert wird resultiert leider in manchen Fällen zu opportunistischen, teilweise tödlichen, Infektionen [176, 177, 181]. Aus diesem Grund wird diese Art der Therapie für Patienten mit chronischen Entzündungen nicht in Frage kommen. Diese Therapie zeigt uns aber, dass chronisch-entzündliche Krankheiten prinzipiell heilbar sind und impliziert, dass vielleicht eine selektive Eliminierung der krankmachenden Komponente des immunologischen Gedächtnisses auch heilend sein kann.

Für eine Komponente, den Plasmazellen, gibt es schon vielversprechende Ansätze, die gezeigt haben, dass die selektive Eliminierung von Plasmazellen einen therapeutischen Nutzen haben könnte [182]. Beim systemischen Lupus erythematodes (SLE), einer primär antikörper-vermittelten Autoimmunkrankheit konnte im Mausmodell [183] und bei refraktären Patienten [184] mittels Elimierung der Plasmazellen mit dem Proteasominhibitor Bortezomib eine signifikante klinische Verbesserung erreicht werden. Allerdings werden mit Bortezomib alle Plasmazellen angegriffen, auch die protektiven [184]. Um eine selektive Eliminierung von autoreaktiven Plasmazellen möglich zu machen, haben wir eine Affinitätsmatrixtechnologie entwickelt, die dies ermöglicht [185]. Dabei wird das Autoantigen über die Affinitätsmatrix auf die Oberfläche an alle Plasmazelle gebracht. Bei den Plasmazellen, die den entsprechenden Autoantikörper sezernieren bindet dieser an das Antigen auf der Oberfläche und tötet die Plasmazelle mittels komplement-vermittelter Lyse [185].

Auch bei Th-Zellen gab es bereits Ansätze durch die Eliminierung von Th-Zellen mit depletierenden anti-CD4-Antikörpern chronisch-entzündliche Krankheiten wie die rheumatoide Arthritis (RA) und Morbus Crohn zu therapieren. Die anfänglichen, Erfolge mit der anti-CD4-Therapie für die RA, die in kleinen *open-label* Studien erzielt wurden [74, 186], ließen sich in größeren, geblindeten, placebo-kontrollierten Studien nicht bestätigen [79, 187]. Auch beim Morbus Crohn gab es widersprüchliche Ergebnisse [77, 188]. Ein Grund für den mangelnden Effekt der CD4-Depletion bei chronischen Entzündungen ist wahrscheinlich die mangelnde Selektivität der Depletion. Durch die CD4-Depletion werden sowohl Effektor-Th-Zellen als auch anti-inflammatorischen regulatorische CD4+ T-Zellen eliminiert. Auch die Th-Lymphopenie, die durch die Therapie induziert wurde und lange nach Absetzen der Therapie anhielt, haben eine weitere klinische Anwendung dieser Art von Therapie verhindert [75, 81]. Eine relativ erfolgreiche Therapie, die auf T-Zellen bei chronischen Entzündungen abzielt, ist die Blockade des kostimulatorischen Moleküls CD80/86 durch CTLA4-Ig (Abatacept). Abatacept bindet mit einer hohen Affinität an CD80/86 auf antigen-präsentierenden Zellen und verhindert die Kostimulation von T-Zellen über CD28 und somit ihre Aktivierung [189]. Bei Patienten mit rheumatoider Arthritis konnte mit Abatacept einer signifikante klinische Verbesserung erreicht

werden [190, 191]. Allerdings unterdrückt Abatacept generell T-Zellantworten und führt zu einer schwächeren protektiven T-zellvermittelten Immunantwort nach Immunisierung [192].

Zur Zeit mangelt es noch an therapeutischen Ansätzen, die selektiv nur die pathogenen Th-Zellen angreifen. Faktoren, wie Twist1 und Hopx bzw. ihre nachgeschalteten Faktoren, wie z.B. miR-148a, die selektiv in mehrfach aktivierten Th1-Zellen stark exprimiert werden, wären mögliche Kandidaten, um pathogene Th-Zellen zu eliminieren. Mögliche Ansätze zum Targeting von microRNAs und intrazellulären Faktoren wären z.B. mit Cholesterolresten modifizierte zell-permeable Antagomire oder siRNAs [193, 194]. Ein alternativer Ansatz wäre die Nutzung von DNAzymen, zellpermable RNA-spezifische Oligonukleotide mit enzymatischer Aktivität. In ersten klinischen Studien wurden Patienten mit asthmatischer Entzündung erfolgreich mit GATA-3-spezifischen DNAzymen behandelt [73].

Schlussendlich ist es aber noch ein langer Weg bis zur selektiven therapeutischen Beeinflussung von chronisch-entzündlichen Erkrankungen, ohne dass dabei das schützende Immunsystem zu beeinträchtigen. Erste Ansätze und Zielstrukturen sind aber vielversprechend und mit einem besseren Verständnis, wie sich das adaptive Immunsystem an chronische Entzündung „adaptiert“, können neue therapeutische Strategien entwickelt werden, die vielleicht auch zur Heilung führen.

5 Zusammenfassung

Th-Lymphozyten spielen aufgrund ihrer Expression von Zytokinen eine zentrale Rolle in der Regulation von Immunantworten. Durch die Zytokine fördern die Th-Zellen die Rekrutierung und Aktivierung von Zellen des angeborenen Immunsystems, den Immunglobulin-Klassenwechsel von B-Zellen und ihre Differenzierung zu antikörper-sezernierenden Plasmazellen. Welche Zytokine eine aktivierte Th-Zelle exprimiert hängt von instruktiven Signalen ab, die sie in der Regel von antigen-präsentierenden Signalen bekommt. Mit den instruktiven Signalen wird ein ganzes Differenzierungsprogramm in den Th-Zellen initiiert, welches am Ende zu einer epigenetischen Prägung des Effektorprogramms führt, sodass die Th-Zelle auch bei nachfolgenden Aktivierungen die gleichen Funktionen ausführt. Während bestimmte Differenzierungsprogramme mit einer effektiven Immunantwort gegen bestimmte Krankheitserreger assoziiert sind – Th1 gegen intrazelluläre Erreger, Th2 gegen Parasiten und Th17 gegen extrazelluläre Bakterien und Pilze – spielen sie gleichermaßen auch eine entscheidende Rolle bei chronisch-entzündlichen Krankheiten. So wird Th1- und Th17-Programmen bei vielen Autoimmunkrankheiten und dem Th2-Programm bei Allergie und asthmatischen Entzündungen eine pathogene Rolle zugeschrieben.

Wir konnten in unseren Arbeiten erstmals zeigen, dass das Th17-Differenzierungsprogramm nicht stabil ist. Th17-Zellen zeichnen sich durch ein hohes Maß an Plastizität aus und können in Gegenwart von Th1-instruierenden Signalen Eigenschaften von Th1-Zellen akquirieren, um zu Th17/Th1-Hybridzellen zu werden. Th17/Th1-Hybridzellen werden insbesondere bei Patienten mit chronisch-entzündlichen Krankheiten und in prä-klinischen Mausmodellen für chronische Entzündung vermehrt detektiert und legen nahe, dass sie eine besondere pathogene Rolle einnehmen. Es ist allerdings noch unklar, ob und wie sie sich in ihrer Fähigkeit Entzündungen auszulösen und aufrechtzuhalten von Th1- und Th17-Zellen unterscheiden. Um dieser Antwort etwas näher zu kommen haben wir den Einfluss der einzelnen Differenzierungsprogramme bei der Entzündungspathologie untersucht. Dabei konnten wir die Rolle vom sogenannten Th1-Mastertranskriptionsfaktor T-bet bei der Regulation der Entzündung im Detail entschlüsseln. Während die Kapazität der Th-Zellen IFN-γ zu exprimineren in der Abwesenheit von T-bet nahezu unbeeinflusst bleibt, ist T-bet essentiell für die Expression von Chemokinen und Chemokinrezeptoren, die Monozyten und Makrophagen und die Th-Zellen selbst an den Ort der Entzündung rekrutieren. Weiterhin wird T-bet in Th-Zellen benötig, um die Differenzierung von Monozyten zu M1 zu fördern. T-bet ist auch ein wichtiger Überlebensfaktor für Th1-Zellen.

In dieser Arbeit konnte auch gezeigt werden, dass die IL-10-Expression, eine wichtige anti-inflammatorische Funktion von Th-Zellen, nicht epigenetisch geprägt ist, im Gegensatz zur IFN-γ-Expression. Dies bedeutet, dass Gedächtnis-Th-Zellen per se erst mal pro-inflammatorisch geprägt sind und nur in Gegenwart instruierender Signale, wie z.B. IL-12, anti-inflammatorisches IL-10 exprimieren. Für die Modulation von T-zellvermittelten chronisch-entzündlichen Krankheiten könnte es daher ein therapeutischer Ansatz sein, IL-10-induzierende Signale zu fördern.

Desweiteren haben wir molekulare Adaptationen identifiziert, die selektiv in chronisch aktivierten Th1-Zellen hochgeregelt werden. Diese Adaptationen, wie die Expression von Twist1 und Hopx, fördern das Überleben der Th1-Zellen am Ort der Entzündung und könnten zur Perpetuation der Entzündung entscheidend beitragen. Für den Transkriptionsfaktor Twist1 haben wir herausgefunden, dass Twist1 das Überleben über die direkte Induktion der miR-148a-Expression steuert. miR-148a inhibiert die Expression des pro-apoptotischen Faktors Bim und reduziert so die Apoptose der Th1-Zellen. Inwiefern miR-148a als direktes therapeutischen Target für die selektive Eliminierung chronisch aktivierter Th1-Zellen geeignet ist, ist Gegenstand aktueller Untersuchungen. Hopx hat keine eigene DNA-Bindeaktivität und muss über die Interaktion mit anderen Partnern seine Funktion ausführen. Mit welchen Partnern Hopx interagiert und wie Hopx das Überleben der Th1-Zellen fördert ist noch unklar und wird derzeit auch von uns untersucht.

Zusammengenommen zeigen unsere Daten, dass Th-Zellen durch ihre pro-inflammatorische Prägung zwar gut gerüstet sind uns gegen Pathogene zu schützen, im Falle einer Autoreaktivität aber auch Immunpathologie und chronische Entzündungen auslösen können. Allerdings weisen unsere Arbeiten auch daraufhin, dass selektive Anpassungen der Th-Zellen an eine chronische Entzündung auch gleichzeitig Ansatzpunkte für ein therapeutisches Eingreifen bei chronisch-entzündlichen Krankheiten darstellen.

6 Referenzen

1 Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. and Coffman, R. L., Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 1986. 136: 2348-2357.

2 Coffman, R. L., Ohara, J., Bond, M. W., Carty, J., Zlotnik, A. and Paul, W. E., B cell stimulatory factor-1 enhances the IgE response of lipopolysaccharide-activated B cells. *J Immunol* 1986. 136: 4538-4541.

3 Lopez, A. F., Sanderson, C. J., Gamble, J. R., Campbell, H. D., Young, I. G. and Vadas, M. A., Recombinant human interleukin 5 is a selective activator of human eosinophil function. *J Exp Med* 1988. 167: 219-224.

4 Harrington, L. E., Mangan, P. R. and Weaver, C. T., Expanding the effector CD4 T-cell repertoire: the Th17 lineage. *Current opinion in immunology* 2006. 18: 349-356.

5 Ivanov, II, Atarashi, K., Manel, N., Brodie, E. L., Shima, T., Karaoz, U., Wei, D., Goldfarb, K. C., Santee, C. A., Lynch, S. V., Tanoue, T., Imaoka, A., Itoh, K., Takeda, K., Umesaki, Y., Honda, K. and Littman, D. R., Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell* 2009. 139: 485-498.

6 Conti, H. R., Shen, F., Nayyar, N., Stocum, E., Sun, J. N., Lindemann, M. J., Ho, A. W., Hai, J. H., Yu, J. J., Jung, J. W., Filler, S. G., Masso-Welch, P., Edgerton, M. and Gaffen, S. L., Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis. *J Exp Med* 2009. 206: 299-311.

7 Dardalhon, V., Awasthi, A., Kwon, H., Galileos, G., Gao, W., Sobel, R. A., Mitsdoerffer, M., Strom, T. B., Elyaman, W., Ho, I. C., Khoury, S., Oukka, M. and Kuchroo, V. K., IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells. *Nat Immunol* 2008. 9: 1347-1355.

8 Veldhoen, M., Uyttenhove, C., van Snick, J., Helmby, H., Westendorf, A., Buer, J., Martin, B., Wilhelm, C. and Stockinger, B., Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat Immunol* 2008. 9: 1341-1346.

9 Eyerich, S., Eyerich, K., Pennino, D., Carbone, T., Nasorri, F., Pallotta, S., Cianfarani, F., Odorisio, T., Traidl-Hoffmann, C., Behrendt, H., Durham, S. R., Schmidt-Weber, C. B. and Cavani, A., Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling. *J Clin Invest* 2009. 119: 3573-3585.

10 Groux, H., O'Garra, A., Bigler, M., Rouleau, M., Antonenko, S., de Vries, J. E. and Roncarolo, M. G., A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 1997. 389: 737-742.

11 Manetti, R., Gerosa, F., Giudizi, M. G., Biagiotti, R., Parronchi, P., Piccinni, M. P., Sampognaro, S., Maggi, E., Romagnani, S., Trinchieri, G. and et al., Interleukin 12 induces stable priming for interferon gamma (IFN-gamma) production during differentiation of human T helper (Th) cells and transient IFN-gamma production in established Th2 cell clones. *The Journal of experimental medicine* 1994. 179: 1273-1283.

12 Seder, R. A., Gazzinelli, R., Sher, A. and Paul, W. E., Interleukin 12 acts directly on CD4+ T cells to enhance priming for interferon gamma production and diminishes interleukin 4 inhibition of such priming. *Proc Natl Acad Sci U S A* 1993. 90: 10188-10192.

13 Schulz, E. G., Mariani, L., Radbruch, A. and Hofer, T., Sequential polarization and imprinting of type 1 T helper lymphocytes by interferon-gamma and interleukin-12. *Immunity* 2009. 30: 673-683.

14 Kopf, M., Le Gros, G., Bachmann, M., Lamers, M. C., Bluethmann, H. and Kohler, G., Disruption of the murine IL-4 gene blocks Th2 cytokine responses. *Nature* 1993. 362: 245-248.

15 Le Gros, G., Ben-Sasson, S. Z., Seder, R., Finkelman, F. D. and Paul, W. E., Generation of interleukin 4 (IL-4)-producing cells in vivo and in vitro: IL-2 and IL-4 are required for in vitro generation of IL-4-producing cells. *J Exp Med* 1990. 172: 921-929.

16 Swain, S., Weinberg, A., English, M. and Huston, G., IL-4 directs the development of Th2-like helper effectors. *J Immunol* 1990. 145: 3796-3806.

17 Mangan, P. R., Harrington, L. E., O'Quinn, D. B., Helms, W. S., Bullard, D. C., Elson, C. O., Hatton, R. D., Wahl, S. M., Schoeb, T. R. and Weaver, C. T., Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature* 2006. 441: 231-234.

18 Santarlasci, V., Maggi, L., Capone, M., Frosali, F., Querci, V., De Palma, R., Liotta, F., Cosmi, L., Maggi, E., Romagnani, S. and Annunziato, F., TGF-beta indirectly favors the development of human Th17 cells by inhibiting Th1 cells. *Eur J Immunol* 2009. 39: 207-215.

19 Korn, T., Mitsdoerffer, M., Croxford, A. L., Awasthi, A., Dardalhon, V. A., Galileos, G., Vollmar, P., Stritesky, G. L., Kaplan, M. H., Waisman, A., Kuchroo, V. K. and Oukka, M., IL-6 controls Th17 immunity in vivo by inhibiting the conversion of conventional T cells into Foxp3+ regulatory T cells. *Proc Natl Acad Sci U S A* 2008. 105: 18460-18465.

20 Veldhoen, M., Hocking, R. J., Atkins, C. J., Locksley, R. M. and Stockinger, B., TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 2006. 24: 179-189.

21 Richter, A., Lohning, M. and Radbruch, A., Instruction for cytokine expression in T helper lymphocytes in relation to proliferation and cell cycle progression. *J Exp Med* 1999. 190: 1439-1450.

22 Grogan, J. L., Mohrs, M., Harmon, B., Lacy, D. A., Sedat, J. W. and Locksley, R. M., Early transcription and silencing of cytokine genes underlie polarization of T helper cell subsets. *Immunity* 2001. 14: 205-215.

23 Kurata, H., Lee, H. J., O'Garra, A. and Arai, N., Ectopic expression of activated Stat6 induces the expression of Th2-specific cytokines and transcription factors in developing Th1 cells. *Immunity* 1999. 11: 677-688.

24 Harrington, L. E., Hatton, R. D., Mangan, P. R., Turner, H., Murphy, T. L., Murphy, K. M. and Weaver, C. T., Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nature immunology* 2005. 6: 1123-1132.

25 Szabo, S. J., Kim, S. T., Costa, G. L., Zhang, X., Fathman, C. G. and Glimcher, L. H., A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 2000. 100: 655-669.

26 Zheng, W. and Flavell, R. A., The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* 1997. 89: 587-596.

27 Ivanov, II, McKenzie, B. S., Zhou, L., Tadokoro, C. E., Lepelley, A., Lafaille, J. J., Cua, D. J. and Littman, D. R., The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* 2006. 126: 1121-1133.

28 Meuer, S. C., Hussey, R. E., Cantrell, D. A., Hodgdon, J. C., Schlossman, S. F., Smith, K. A. and Reinherz, E. L., Triggering of the T3-Ti antigen-receptor complex results in clonal T-cell proliferation through an interleukin 2-dependent autocrine pathway. *Proc Natl Acad Sci U S A* 1984. 81: 1509-1513.

29 Assenmacher, M., Lohning, M., Scheffold, A., Manz, R. A., Schmitz, J. and Radbruch, A., Sequential production of IL-2, IFN-gamma and IL-10 by individual staphylococcal enterotoxin B-activated T helper lymphocytes. *Eur J Immunol* 1998. 28: 1534-1543.

30 Lohning, M., Richter, A. and Radbruch, A., Cytokine memory of T helper lymphocytes. *Adv Immunol* 2002. 80: 115-181.

31 Lohning, M., Richter, A., Stamm, T., Hu-Li, J., Assenmacher, M., Paul, W. E. and Radbruch, A., Establishment of memory for IL-10 expression in developing T helper 2 cells requires repetitive IL-4 costimulation and does not impair proliferation. *Proc Natl Acad Sci U S A* 2003. 100: 12307-12312.

32 Probst, A. V., Dunleavy, E. and Almouzni, G., Epigenetic inheritance during the cell cycle. *Nat Rev Mol Cell Biol* 2009. 10: 192-206.

33 Cohen, S. M., Chastain, P. D., 2nd, Rosson, G. B., Groh, B. S., Weissman, B. E., Kaufman, D. G. and Bultman, S. J., BRG1 co-localizes with DNA replication factors and is required for efficient replication fork progression. *Nucleic Acids Res* 2010. 38: 6906-6919.

34 Zhang, F. and Boothby, M., T helper type 1-specific Brg1 recruitment and remodeling of nucleosomes positioned at the IFN-gamma promoter are Stat4 dependent. *The Journal of experimental medicine* 2006. 203: 1493-1505.

35 Wurster, A. L. and Pazin, M. J., BRG1-mediated chromatin remodeling regulates differentiation and gene expression of T helper cells. *Mol Cell Biol* 2008. 28: 7274-7285.

36 Agarwal, S. and Rao, A., Modulation of chromatin structure regulates cytokine gene expression during T cell differentiation. *Immunity* 1998. 9: 765-775.

37 Fields, P. E., Kim, S. T. and Flavell, R. A., Cutting edge: changes in histone acetylation at the IL-4 and IFN-gamma loci accompany Th1/Th2 differentiation. *J Immunol* 2002. 169: 647-650.

38 Tykocinski, L. O., Hajkova, P., Chang, H. D., Stamm, T., Sozeri, O., Lohning, M., Hu-Li, J., Niesner, U., Kreher, S., Friedrich, B., Pannetier, C., Grutz, G., Walter, J., Paul, W. E. and Radbruch, A., A critical control element for interleukin-4 memory expression in T helper lymphocytes. *J Biol Chem* 2005. 280: 28177-28185.

39 Ansel, K. M., Lee, D. U. and Rao, A., An epigenetic view of helper T cell differentiation. *Nat Immunol* 2003. 4: 616-623.

40 O'Shea, J. J., Lahesmaa, R., Vahedi, G., Laurence, A. and Kanno, Y., Genomic views of STAT function in CD4+ T helper cell differentiation. *Nat Rev Immunol* 2011. 11: 239-250.

41 Wei, L., Vahedi, G., Sun, H. W., Watford, W. T., Takatori, H., Ramos, H. L., Takahashi, H., Liang, J., Gutierrez-Cruz, G., Zang, C., Peng, W., O'Shea, J. J. and Kanno, Y., Discrete roles of STAT4 and STAT6 transcription factors in tuning epigenetic modifications and transcription during T helper cell differentiation. *Immunity* 2010. 32: 840-851.

42 Takemoto, N., Kamogawa, Y., Jun Lee, H., Kurata, H., Arai, K. I., O'Garra, A., Arai, N. and Miyatake, S., Cutting edge: chromatin remodeling at the IL-4/IL-13 intergenic regulatory region for Th2-specific cytokine gene cluster. *J Immunol* 2000. 165: 6687-6691.

43 Lee, H. J., Takemoto, N., Kurata, H., Kamogawa, Y., Miyatake, S., O'Garra, A. and Arai, N., GATA-3 induces T helper cell type 2 (Th2) cytokine expression and chromatin remodeling in committed Th1 cells. *J Exp Med* 2000. 192: 105-115.

44 Ouyang, W., Lohning, M., Gao, Z., Assenmacher, M., Ranganath, S., Radbruch, A. and Murphy, K. M., Stat6-independent GATA-3 autoactivation directs IL-4-independent Th2 development and commitment. *Immunity* 2000. 12: 27-37.

45 Asnagli, H., Afkarian, M. and Murphy, K. M., Cutting edge: Identification of an alternative GATA-3 promoter directing tissue-specific gene expression in mouse and human. *J Immunol* 2002. 168: 4268-4271.

46 Hofer, T., Nathansen, H., Lohning, M., Radbruch, A. and Heinrich, R., GATA-3 transcriptional imprinting in Th2 lymphocytes: a mathematical model. *Proc Natl Acad Sci U S A* 2002. 99: 9364-9368.

47 Zhu, J., Min, B., Hu-Li, J., Watson, C. J., Grinberg, A., Wang, Q., Killeen, N., Urban, J. F., Jr., Guo, L. and Paul, W. E., Conditional deletion of Gata3 shows its essential function in T(H)1-T(H)2 responses. *Nat Immunol* 2004. 5: 1157-1165.

48 Afkarian, M., Sedy, J. R., Yang, J., Jacobson, N. G., Cereb, N., Yang, S. Y., Murphy, T. L. and Murphy, K. M., T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells. *Nat Immunol* 2002. 3: 549-557.

49 Zhu, J., Jankovic, D., Oler, A. J., Wei, G., Sharma, S., Hu, G., Guo, L., Yagi, R., Yamane, H., Punkosdy, G., Feigenbaum, L., Zhao, K. and Paul, W. E., The transcription factor T-bet is induced by multiple pathways and prevents an endogenous Th2 cell program during Th1 cell responses. *Immunity* 2012. 37: 660-673.

50 Mullen, A. C., Hutchins, A. S., High, F. A., Lee, H. W., Sykes, K. J., Chodosh, L. A. and Reiner, S. L., Hlx is induced by and genetically interacts with T-bet to promote heritable T(H)1 gene induction. *Nat Immunol* 2002. 3: 652-658.

51 Djuretic, I. M., Levanon, D., Negreanu, V., Groner, Y., Rao, A. and Ansel, K. M., Transcription factors T-bet and Runx3 cooperate to activate Ifng and silence Il4 in T helper type 1 cells. *Nature immunology* 2007. 8: 145-153.

52 Akimzhanov, A. M., Yang, X. O. and Dong, C., Chromatin remodeling of interleukin-17 (IL-17)-IL-17F cytokine gene locus during inflammatory helper T cell differentiation. *The Journal of biological chemistry* 2007. 282: 5969-5972.

53 Mukasa, R., Balasubramani, A., Lee, Y. K., Whitley, S. K., Weaver, B. T., Shibata, Y., Crawford, G. E., Hatton, R. D. and Weaver, C. T., Epigenetic instability of cytokine and transcription factor gene loci underlies plasticity of the T helper 17 cell lineage. *Immunity* 2010. 32: 616-627.

54 Nurieva, R., Yang, X. O., Martinez, G., Zhang, Y., Panopoulos, A. D., Ma, L., Schluns, K., Tian, Q., Watowich, S. S., Jetten, A. M. and Dong, C., Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature* 2007. 448: 480-483.

55 Zhou, L., Ivanov, II, Spolski, R., Min, R., Shenderov, K., Egawa, T., Levy, D. E., Leonard, W. J. and Littman, D. R., IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat Immunol* 2007. 8: 967-974.

56 Korn, T., Bettelli, E., Gao, W., Awasthi, A., Jager, A., Strom, T. B., Oukka, M. and Kuchroo, V. K., IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. *Nature* 2007. 448: 484-487.

57 Peguet-Navarro, J., Moulon, C., Caux, C., Dalbiez-Gauthier, C., Banchereau, J. and Schmitt, D., Inhibitory effect of IL-10 on human Langerhans cell antigen presenting function. *Adv Exp Med Biol* 1995. 378: 359-361.

58 Flores Villanueva, P. O., Reiser, H. and Stadecker, M. J., Regulation of T helper cell responses in experimental murine schistosomiasis by IL-10. Effect on expression of B7 and B7-2 costimulatory molecules by macrophages. *J Immunol* 1994. 153: 5190-5199.

59 Ralph, P., Nakoinz, I., Sampson-Johannes, A., Fong, S., Lowe, D., Min, H. Y. and Lin, L., IL-10, T lymphocyte inhibitor of human blood cell production of IL-1 and tumor necrosis factor. *J Immunol* 1992. 148: 808-814.

60 Fiorentino, D. F., Zlotnik, A., Mosmann, T. R., Howard, M. and O'Garra, A., IL-10 inhibits cytokine production by activated macrophages. *J Immunol* 1991. 147: 3815-3822.

61 Fiorentino, D. F., Zlotnik, A., Vieira, P., Mosmann, T. R., Howard, M., Moore, K. W. and O'Garra, A., IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J Immunol* 1991. 146: 3444-3451.

62 Itoh, K. and Hirohata, S., The role of IL-10 in human B cell activation, proliferation, and differentiation. *J Immunol* 1995. 154: 4341-4350.

63 Heine, G., Drozdenko, G., Grun, J. R., Chang, H. D., Radbruch, A. and Worm, M., Autocrine IL-10 promotes human B-cell differentiation into IgM- or IgG-secreting plasmablasts. *Eur J Immunol* 2014. 44: 1615-1621.

64 Kühn, R., Löhler, J., Rennick, D., Rajewsky, K. and Müller, W., Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 1993. 75: 263-274.

65 Roers, A., Siewe, L., Strittmatter, E., Deckert, M., Schluter, D., Stenzel, W., Gruber, A. D., Krieg, T., Rajewsky, K. and Muller, W., T cell-specific inactivation of the interleukin 10 gene in mice results in enhanced T cell responses but normal innate responses to lipopolysaccharide or skin irritation. *The Journal of experimental medicine* 2004. 200: 1289-1297.

66 Chang, H. D., Helbig, C., Tykocinski, L., Kreher, S., Koeck, J., Niesner, U. and Radbruch, A., Expression of IL-10 in Th memory lymphocytes is conditional on IL-12 or IL-4, unless the IL-10 gene is imprinted by GATA-3. *Eur J Immunol* 2007. 37: 807-817.

67 Zielinski, C. E., Mele, F., Aschenbrenner, D., Jarrossay, D., Ronchi, F., Gattorno, M., Monticelli, S., Lanzavecchia, A. and Sallusto, F., Pathogen-induced human TH17 cells produce IFN-gamma or IL-10 and are regulated by IL-1beta. *Nature* 2012. 484: 514-518.

68 Shoemaker, J., Saraiva, M. and O'Garra, A., GATA-3 Directly Remodels the IL-10 Locus Independently of IL-4 in CD4+ T Cells. *J Immunol* 2006. 176: 3470-3479.

69 Rutz, S., Janke, M., Kassner, N., Hohnstein, T., Krueger, M. and Scheffold, A., Notch regulates IL-10 production by T helper 1 cells. *Proceedings of the National Academy of Sciences of the United States of America* 2008. 105: 3497-3502.

70 Sadick, M. D., Locksley, R. M., Tubbs, C. and Raff, H. V., Murine cutaneous leishmaniasis: resistance correlates with the capacity to generate interferon-gamma in response to Leishmania antigens in vitro. *J Immunol* 1986. 136: 655-661.

71 Yamamura, M., Uyemura, K., Deans, R. J., Weinberg, K., Rea, T. H., Bloom, B. R. and Modlin, R. L., Defining protective responses to pathogens: cytokine profiles in leprosy lesions. *Science* 1991. 254: 277-279.

72 Zhang, D. H., Yang, L., Cohn, L., Parkyn, L., Homer, R., Ray, P. and Ray, A., Inhibition of allergic inflammation in a murine model of asthma by expression of a dominant-negative mutant of GATA-3. *Immunity* 1999. 11: 473-482.

73 Krug, N., Hohlfeld, J. M., Kirsten, A. M., Kornmann, O., Beeh, K. M., Kappeler, D., Korn, S., Ignatenko, S., Timmer, W., Rogon, C., Zeitvogel, J., Zhang, N., Bille, J., Homburg, U., Turowska, A., Bachert, C., Werfel, T., Buhl, R., Renz, J., Garn, H. and Renz, H., Allergen-induced asthmatic responses modified by a GATA3-specific DNAzyme. *N Engl J Med* 2015. 372: 1987-1995.

74 Horneff, G., Burmester, G. R., Emmrich, F. and Kalden, J. R., Treatment of rheumatoid arthritis with an anti-CD4 monoclonal antibody. *Arthritis and rheumatism* 1991. 34: 129-140.

75 Moreland, L. W., Pratt, P. W., Bucy, R. P., Jackson, B. S., Feldman, J. W. and Koopman, W. J., Treatment of refractory rheumatoid arthritis with a chimeric anti-CD4 monoclonal antibody. Long-term followup of CD4+ T cell counts. *Arthritis Rheum* 1994. 37: 834-838.

76 van der Lubbe, P. A., Reiter, C., Breedveld, F. C., Kruger, K., Schattenkirchner, M., Sanders, M. E. and Riethmuller, G., Chimeric CD4 monoclonal antibody cM-T412 as a therapeutic approach to rheumatoid arthritis. *Arthritis Rheum* 1993. 36: 1375-1379.

77 Stronkhorst, A., Radema, S., Yong, S. L., Bijl, H., ten Berge, I. J., Tytgat, G. N. and van Deventer, S. J., CD4 antibody treatment in patients with active Crohn's disease: a phase 1 dose finding study. *Gut* 1997. 40: 320-327.

78 van der Lubbe, P. A., Dijkmans, B. A., Markusse, H. M., Nassander, U. and Breedveld, F. C., A randomized, double-blind, placebo-controlled study of CD4 monoclonal antibody therapy in early rheumatoid arthritis. *Arthritis Rheum* 1995. 38: 1097-1106.

79 Moreland, L. W., Pratt, P. W., Mayes, M. D., Postlethwaite, A., Weisman, M. H., Schnitzer, T., Lightfoot, R., Calabrese, L., Zelinger, D. J., Woody, J. N. and et al., Double-blind, placebo-controlled multicenter trial using chimeric monoclonal anti-CD4 antibody, cM-T412, in rheumatoid arthritis patients receiving concomitant methotrexate. *Arthritis Rheum* 1995. 38: 1581-1588.

80 Schulze-Koops, H. and Lipsky, P. E., Anti-CD4 monoclonal antibody therapy in human autoimmune diseases. *Curr Dir Autoimmun* 2000. 2: 24-49.

81 Horneff, G., Emmrich, F., Reiter, C., Kalden, J. R. and Burmester, G. R., Persistent depletion of CD4+ T cells and inversion of the CD4/CD8 T cell ratio induced by anti-CD4 therapy. *The Journal of rheumatology* 1992. 19: 1845-1850.

82 Powrie, F., Leach, M. W., Mauze, S., Caddle, L. B. and Coffman, R. L., Phenotypically distinct subsets of CD4+ T cells induce or protect from chronic intestinal inflammation in C. B-17 scid mice. *Int Immunol* 1993. 5: 1461-1471.

83 Katz, J. D., Benoist, C. and Mathis, D., T helper cell subsets in insulin-dependent diabetes. *Science* 1995. 268: 1185-1188.

84 Ben-Nun, A. and Lando, Z., Detection of autoimmune cells proliferating to myelin basic protein and selection of T cell lines that mediate experimental autoimmune encephalomyelitis (EAE) in mice. *Journal of immunology (Baltimore, Md.* 1983. 130: 1205-1209.

85 Klasen, I. S., Donselaar, I. G., Ladestein, R. M., van den Berg, W. B. and Benner, R., Joint inflammations and flare-up reactions in mice induced by a helper T cell clone. *Agents and actions* 1986. 19: 331-334.

86 Oppmann, B., Lesley, R., Blom, B., Timans, J. C., Xu, Y., Hunte, B., Vega, F., Yu, N., Wang, J., Singh, K., Zonin, F., Vaisberg, E., Churakova, T., Liu, M., Gorman, D., Wagner, J., Zurawski, S., Liu, Y., Abrams, J. S., Moore, K. W., Rennick, D., de Waal-Malefyt, R., Hannum, C., Bazan, J. F. and Kastelein, R. A., Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* 2000. 13: 715-725.

87 Leonard, J. P., Waldburger, K. E. and Goldman, S. J., Prevention of experimental autoimmune encephalomyelitis by antibodies against interleukin 12. *The Journal of experimental medicine* 1995. 181: 381-386.

88 Paola Zaccone, P. H. F. N. G. P. L. A. A. C., The involvement of IL-12 in murine experimentally induced autoimmune thyroid disease. *European journal of immunology* 1999. 29: 1933-1942.

89 McIntyre, K. W., Shuster, D. J., Gillooly, K. M., Warrier, R. R., Connaughton, S. E., Hall, L. B., Arp, L. H., Gately, M. K. and Magram, J., Reduced incidence and severity of collagen-induced arthritis in interleukin-12-deficient mice. *European journal of immunology* 1996. 26: 2933-2938.

90 Murphy, C. A., Langrish, C. L., Chen, Y., Blumenschein, W., McClanahan, T., Kastelein, R. A., Sedgwick, J. D. and Cua, D. J., Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. *The Journal of experimental medicine* 2003. 198: 1951-1957.

91 Neurath, M. F., Weigmann, B., Finotto, S., Glickman, J., Nieuwenhuis, E., Iijima, H., Mizoguchi, A., Mizoguchi, E., Mudter, J., Galle, P. R., Bhan, A., Autschbach, F., Sullivan, B. M., Szabo, S. J., Glimcher, L. H. and Blumberg, R. S., The Transcription Factor T-bet Regulates Mucosal T Cell Activation in Experimental Colitis and Crohn's Disease. *J. Exp. Med.* 2002. 195: 1129-1143.

92 Codarri, L., Gyulveszi, G., Tosevski, V., Hesske, L., Fontana, A., Magnenat, L., Suter, T. and Becher, B., RORgammat drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuroinflammation. *Nat Immunol* 2011. 12: 560-567.

93 Park, H., Li, Z., Yang, X. O., Chang, S. H., Nurieva, R., Wang, Y. H., Wang, Y., Hood, L., Zhu, Z., Tian, Q. and Dong, C., A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nature immunology* 2005. 6: 1133-1141.

94 Annunziato, F., Cosmi, L., Santarlasci, V., Maggi, L., Liotta, F., Mazzinghi, B., Parente, E., Fili, L., Ferri, S., Frosali, F., Giudici, F., Romagnani, P., Parronchi, P., Tonelli, F., Maggi, E. and Romagnani, S., Phenotypic and functional features of human Th17 cells. *The Journal of experimental medicine* 2007. 204: 1849-1861.

95 Kebir, H., Ifergan, I., Alvarez, J. I., Bernard, M., Poirier, J., Arbour, N., Duquette, P. and Prat, A., Preferential recruitment of interferon-gamma-expressing T H 17 cells in multiple sclerosis. *Ann Neurol* 2009. 66: 390-402.

96 O'Connor, R. A., Prendergast, C. T., Sabatos, C. A., Lau, C. W., Leech, M. D., Wraith, D. C. and Anderton, S. M., Cutting edge: Th1 cells facilitate the entry of Th17 cells to the central nervous system during experimental autoimmune encephalomyelitis. *Journal of immunology (Baltimore, Md.* 2008. 181: 3750-3754.

97 Lees, J. R., Iwakura, Y. and Russell, J. H., Host T cells are the main producers of IL-17 within the central nervous system during initiation of experimental autoimmune encephalomyelitis induced by adoptive transfer of Th1 cell lines. *Journal of immunology (Baltimore, Md.* 2008. 180: 8066-8072.

98 Muljo, S. A., Ansel, K. M., Kanellopoulou, C., Livingston, D. M., Rao, A. and Rajewsky, K., Aberrant T cell differentiation in the absence of Dicer. *The Journal of experimental medicine* 2005. 202: 261-269.

99 Mosmann, T. R. and Coffman, R. L., TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 1989. 7: 145-173.

100 Metwali, A., Blum, A., Elliott, D. E. and Weinstock, J. V., Interleukin-4 receptor alpha chain and STAT6 signaling inhibit gamma interferon but not Th2 cytokine expression within schistosome granulomas. *Infect Immun* 2002. 70: 5651-5658.

101 Usui, T., Nishikomori, R., Kitani, A. and Strober, W., GATA-3 suppresses Th1 development by downregulation of Stat4 and not through effects on IL-12Rbeta2 chain or T-bet. *Immunity* 2003. 18: 415-428.

102 Hwang, E. S., Szabo, S. J., Schwartzberg, P. L. and Glimcher, L. H., T helper cell fate specified by kinase-mediated interaction of T-bet with GATA-3. *Science* 2005. 307: 430-433.

103 Jenner, R. G., Townsend, M. J., Jackson, I., Sun, K., Bouwman, R. D., Young, R. A., Glimcher, L. H. and Lord, G. M., The transcription factors T-bet and GATA-3 control alternative pathways of T-cell differentiation through a shared set of target genes. *Proc Natl Acad Sci U S A* 2009. 106: 17876-17881.

104 Ansel, K. M., Greenwald, R. J., Agarwal, S., Bassing, C. H., Monticelli, S., Interlandi, J., Djuretic, I. M., Lee, D. U., Sharpe, A. H., Alt, F. W. and Rao, A., Deletion of a conserved Il4 silencer impairs T helper type 1-mediated immunity. *Nat Immunol* 2004. 5: 1251-1259.

105 Chang, S. and Aune, T. M., Dynamic changes in histone-methylation 'marks' across the locus encoding interferon-gamma during the differentiation of T helper type 2 cells. *Nat Immunol* 2007. 8: 723-731.

106 Kanhere, A., Hertweck, A., Bhatia, U., Gokmen, M. R., Perucha, E., Jackson, I., Lord, G. M. and Jenner, R. G., T-bet and GATA3 orchestrate Th1 and Th2 differentiation through lineage-specific targeting of distal regulatory elements. *Nat Commun* 2012. 3: 1268.

107 Janson, P. C., Linton, L. B., Bergman, E. A., Marits, P., Eberhardson, M., Piehl, F., Malmstrom, V. and Winqvist, O., Profiling of CD4+ T cells with epigenetic immune lineage analysis. *J Immunol* 2011. 186: 92-102.

108 Lee, Y. K., Turner, H., Maynard, C. L., Oliver, J. R., Chen, D., Elson, C. O. and Weaver, C. T., Late developmental plasticity in the T helper 17 lineage. *Immunity* 2009. 30: 92-107.

109 Brugnolo, F., Sampognaro, S., Liotta, F., Cosmi, L., Annunziato, F., Manuelli, C., Campi, P., Maggi, E., Romagnani, S. and Parronchi, P., The novel synthetic immune response modifier R-848 (Resiquimod) shifts human allergen-specific CD4+ TH2 lymphocytes into IFN-gamma-producing cells. *J Allergy Clin Immunol* 2003. 111: 380-388.

110 Hegazy, A. N., Peine, M., Helmstetter, C., Panse, I., Frohlich, A., Bergthaler, A., Flatz, L., Pinschewer, D. D., Radbruch, A. and Lohning, M., Interferons direct Th2 cell reprogramming to generate a stable GATA-3(+)T-bet(+) cell subset with combined Th2 and Th1 cell functions. *Immunity* 2010. 32: 116-128.

111 Lexberg, M. H., Taubner, A., Albrecht, I., Lepenies, I., Richter, A., Kamradt, T., Radbruch, A. and Chang, H. D., IFN-gamma and IL-12 synergize to convert in vivo generated Th17 into Th1/Th17 cells. *Eur J Immunol* 2010. 40: 3017-3027.

112 Zimmermann, J., Kuhl, A. A., Weber, M., Grun, J. R., Loffler, J., Haftmann, C., Riedel, R., Maschmeyer, P., Lehmann, K., Westendorf, K., Mashreghi, M. F., Lohning, M., Mack, M., Radbruch, A. and Chang, H. D., T-bet expression by Th cells promotes type 1 inflammation but is dispensable for colitis. *Mucosal Immunol* 2016. 9: 1487-1499.

113 Peine, M., Rausch, S., Helmstetter, C., Frohlich, A., Hegazy, A. N., Kuhl, A. A., Grevelding, C. G., Hofer, T., Hartmann, S. and Lohning, M., Stable T-bet(+)GATA-3(+) Th1/Th2 hybrid cells arise in vivo, can develop directly from naive precursors, and limit immunopathologic inflammation. *PLoS Biol* 2013. 11: e1001633.

114 Dong, J., Ivascu, C., Chang, H. D., Wu, P., Angeli, R., Maggi, L., Eckhardt, F., Tykocinski, L., Haefliger, C., Mowes, B., Sieper, J., Radbruch, A., Annunziato, F. and Thiel, A., IL-10 is excluded from the functional cytokine memory of human CD4+ memory T lymphocytes. *J Immunol* 2007. 179: 2389-2396.

115 Dong, J., Chang, H. D., Ivascu, C., Qian, Y., Rezai, S., Okhrimenko, A., Cosmi, L., Maggi, L., Eckhardt, F., Wu, P., Sieper, J., Alexander, T., Annunziato, F., Gossen, M., Li, J., Radbruch, A. and Thiel, A., Loss of methylation at the IFNG promoter and CNS-1 is

associated with the development of functional IFN-gamma memory in human CD4(+) T lymphocytes. *Eur J Immunol* 2013. 43: 793-804.

116 Janke, M., Peine, M., Nass, A., Morawietz, L., Hamann, A. and Scheffold, A., In-vitro-induced Th17 cells fail to induce inflammation in vivo and show an impaired migration into inflamed sites. *Eur J Immunol* 2010. 40: 1089-1098.

117 Bending, D., De la Pena, H., Veldhoen, M., Phillips, J. M., Uyttenhove, C., Stockinger, B. and Cooke, A., Highly purified Th17 cells from BDC2.5NOD mice convert into Th1-like cells in NOD/SCID recipient mice. *J Clin Invest* 2009. 119: 565-572.

118 Lexberg, M. H., Taubner, A., Forster, A., Albrecht, I., Richter, A., Kamradt, T., Radbruch, A. and Chang, H. D., Th memory for interleukin-17 expression is stable in vivo. *Eur J Immunol* 2008. 38: 2654-2664.

119 Mullen, A. C., High, F. A., Hutchins, A. S., Lee, H. W., Villarino, A. V., Livingston, D. M., Kung, A. L., Cereb, N., Yao, T. P., Yang, S. Y. and Reiner, S. L., Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. *Science* 2001. 292: 1907-1910.

120 Maggi, L., Santarlasci, V., Capone, M., Rossi, M. C., Querci, V., Mazzoni, A., Cimaz, R., De Palma, R., Liotta, F., Maggi, E., Romagnani, S., Cosmi, L. and Annunziato, F., Distinctive features of classic and nonclassic (Th17 derived) human Th1 cells. *Eur J Immunol* 2012. 42: 3180-3188.

121 Maggi, L., Capone, M., Giudici, F., Santarlasci, V., Querci, V., Liotta, F., Ficari, F., Maggi, E., Tonelli, F., Annunziato, F. and Cosmi, L., CD4+CD161+ T lymphocytes infiltrate Crohn's disease-associated perianal fistulas and are reduced by anti-TNF-alpha local therapy. *Int Arch Allergy Immunol* 2013. 161: 81-86.

122 Cosmi, L., Cimaz, R., Maggi, L., Santarlasci, V., Capone, M., Borriello, F., Frosali, F., Querci, V., Simonini, G., Barra, G., Piccinni, M. P., Liotta, F., De Palma, R., Maggi, E., Romagnani, S. and Annunziato, F., Evidence of the transient nature of the Th17 phenotype of CD4+CD161+ T cells in the synovial fluid of patients with juvenile idiopathic arthritis. *Arthritis Rheum* 2011. 63: 2504-2515.

123 Mazzoni, A., Santarlasci, V., Maggi, L., Capone, M., Rossi, M. C., Querci, V., De Palma, R., Chang, H. D., Thiel, A., Cimaz, R., Liotta, F., Cosmi, L., Maggi, E., Radbruch, A., Romagnani, S., Dong, J. and Annunziato, F., Demethylation of the RORC2 and IL17A in human CD4+ T lymphocytes defines Th17 origin of nonclassic Th1 cells. *J Immunol* 2015. 194: 3116-3126.

124 Martin-Orozco, N., Chung, Y., Chang, S. H., Wang, Y. H. and Dong, C., Th17 cells promote pancreatic inflammation but only induce diabetes efficiently in lymphopenic hosts after conversion into Th1 cells. *Eur J Immunol* 2009. 39: 216-224.

125 Hirota, K., Duarte, J. H., Veldhoen, M., Hornsby, E., Li, Y., Cua, D. J., Ahlfors, H., Wilhelm, C., Tolaini, M., Menzel, U., Garefalaki, A., Potocnik, A. J. and Stockinger, B., Fate mapping of IL-17-producing T cells in inflammatory responses. *Nat Immunol* 2011. 12: 255-263.

126 Morrison, P. J., Bending, D., Fouser, L. A., Wright, J. F., Stockinger, B., Cooke, A. and Kullberg, M. C., Th17-cell plasticity in Helicobacter hepaticus-induced intestinal inflammation. *Mucosal Immunol* 2013. 6: 1143-1156.

127 Pepper, M., Linehan, J. L., Pagan, A. J., Zell, T., Dileepan, T., Cleary, P. P. and Jenkins, M. K., Different routes of bacterial infection induce long-lived TH1 memory cells and short-lived TH17 cells. *Nat Immunol* 2010. 11: 83-89.

128 Liu, H. P., Cao, A. T., Feng, T., Li, Q., Zhang, W., Yao, S., Dann, S. M., Elson, C. O. and Cong, Y., TGF-beta converts Th1 cells into Th17 cells through stimulation of Runx1 expression. *Eur J Immunol* 2015. 45: 1010-1018.

129 Ghoreschi, K., Laurence, A., Yang, X. P., Tato, C. M., McGeachy, M. J., Konkel, J. E., Ramos, H. L., Wei, L., Davidson, T. S., Bouladoux, N., Grainger, J. R., Chen, Q., Kanno, Y., Watford, W. T., Sun, H. W., Eberl, G., Shevach, E. M., Belkaid, Y., Cua, D. J., Chen, W. and O'Shea, J. J., Generation of pathogenic T(H)17 cells in the absence of TGF-beta signalling. *Nature* 2010. 467: 967-971.

130 Lee, Y., Awasthi, A., Yosef, N., Quintana, F. J., Xiao, S., Peters, A., Wu, C., Kleinewietfeld, M., Kunder, S., Hafler, D. A., Sobel, R. A., Regev, A. and Kuchroo, V. K., Induction and molecular signature of pathogenic TH17 cells. *Nat Immunol* 2012. 13: 991-999.

131 Haftmann, C., Stittrich, A. B., Zimmermann, J., Fang, Z., Hradilkova, K., Bardua, M., Westendorf, K., Heinz, G. A., Riedel, R., Siede, J., Lehmann, K., Weinberger, E. E., Zimmel, D., Lauer, U., Haupl, T., Sieper, J., Backhaus, M., Neumann, C., Hoffmann, U., Porstner, M., Chen, W., Grun, J. R., Baumgrass, R., Matz, M., Lohning, M., Scheffold, A., Wittmann, J., Chang, H. D., Rajewsky, N., Jack, H. M., Radbruch, A. and Mashreghi, M. F., miR-148a is upregulated by Twist1 and T-bet and promotes Th1-cell survival by regulating the proapoptotic gene Bim. *Eur J Immunol* 2014.

132 Papadakis, K. A., Prehn, J., Nelson, V., Cheng, L., Binder, S. W., Ponath, P. D., Andrew, D. P. and Targan, S. R., The role of thymus-expressed chemokine and its receptor CCR9 on lymphocytes in the regional specialization of the mucosal immune system. *J Immunol* 2000. 165: 5069-5076.

133 Wang, C., Kang, S. G., Lee, J., Sun, Z. and Kim, C. H., The roles of CCR6 in migration of Th17 cells and regulation of effector T-cell balance in the gut. *Mucosal Immunol* 2009. 2: 173-183.

134 Papadakis, K. A., Prehn, J., Zhu, D., Landers, C., Gaiennie, J., Fleshner, P. R. and Targan, S. R., Expression and regulation of the chemokine receptor CXCR3 on lymphocytes from normal and inflammatory bowel disease mucosa. *Inflammatory bowel diseases* 2004. 10: 778-788.

135 Bonecchi, R., Bianchi, G., Bordignon, P. P., D'Ambrosio, D., Lang, R., Borsatti, A., Sozzani, S., Allavena, P., Gray, P. A., Mantovani, A. and Sinigaglia, F., Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J Exp Med* 1998. 187: 129-134.

136 Mills, C. D., Kincaid, K., Alt, J. M., Heilman, M. J. and Hill, A. M., M-1/M-2 macrophages and the Th1/Th2 paradigm. *J Immunol* 2000. 164: 6166-6173.

137 Targan, S. R., Hanauer, S. B., van Deventer, S. J., Mayer, L., Present, D. H., Braakman, T., DeWoody, K. L., Schaible, T. F. and Rutgeerts, P. J., A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor alpha for Crohn's disease. Crohn's Disease cA2 Study Group. *N Engl J Med* 1997. 337: 1029-1035.

138 Hommes, D. W., Mikhajlova, T. L., Stoinov, S., Stimac, D., Vucelic, B., Lonovics, J., Zakuciova, M., D'Haens, G., Van Assche, G., Ba, S., Lee, S. and Pearce, T., Fontolizumab, a humanised anti-interferon gamma antibody, demonstrates safety and clinical activity in patients with moderate to severe Crohn's disease. *Gut* 2006. 55: 1131-1137.

139 Sandborn, W. J., Gasink, C., Gao, L. L., Blank, M. A., Johanns, J., Guzzo, C., Sands, B. E., Hanauer, S. B., Targan, S., Rutgeerts, P., Ghosh, S., de Villiers, W. J., Panaccione, R., Greenberg, G., Schreiber, S., Lichtiger, S., Feagan, B. G. and Group, C. S., Ustekinumab induction and maintenance therapy in refractory Crohn's disease. *N Engl J Med* 2012. 367: 1519-1528.

140 Sandborn, W. J., Feagan, B. G., Rutgeerts, P., Hanauer, S., Colombel, J. F., Sands, B. E., Lukas, M., Fedorak, R. N., Lee, S., Bressler, B., Fox, I., Rosario, M., Sankoh, S., Xu, J., Stephens, K., Milch, C., Parikh, A. and Group, G. S., Vedolizumab as induction and maintenance therapy for Crohn's disease. *N Engl J Med* 2013. 369: 711-721.

141 Asseman, C., Read, S. and Powrie, F., Colitogenic Th1 Cells Are Present in the Antigen-Experienced T Cell Pool in Normal Mice: Control by CD4+ Regulatory T Cells and IL-10. *J Immunol* 2003. 171: 971-978.

142 McGeachy, M. J., Bak-Jensen, K. S., Chen, Y., Tato, C. M., Blumenschein, W., McClanahan, T. and Cua, D. J., TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology. *Nature immunology* 2007. 8: 1390-1397.

143 Rubtsov, Y. P., Rasmussen, J. P., Chi, E. Y., Fontenot, J., Castelli, L., Ye, X., Treuting, P., Siewe, L., Roers, A., Henderson, W. R., Jr., Muller, W. and Rudensky, A. Y., Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity* 2008. 28: 546-558.

144 Belkaid, Y., Hoffmann, K. F., Mendez, S., Kamhawi, S., Udey, M. C., Wynn, T. A. and Sacks, D. L., The role of interleukin (IL)-10 in the persistence of Leishmania major in the skin after healing and the therapeutic potential of anti-IL-10 receptor antibody for sterile cure. *J Exp Med* 2001. 194: 1497-1506.

145 Anderson, C. F., Oukka, M., Kuchroo, V. J. and Sacks, D., CD4+CD25-Foxp3- Th1 cells are the source of IL-10-mediated immune suppression in chronic cutaneous leishmaniasis. *J. Exp. Med.* 2007: jem.20061886.

146 Jankovic, D., Kullberg, M. C., Feng, C. G., Goldszmid, R. S., Collazo, C. M., Wilson, M., Wynn, T. A., Kamanaka, M., Flavell, R. A. and Sher, A., Conventional T-bet+Foxp3- Th1 cells are the major source of host-protective regulatory IL-10 during intracellular protozoan infection 2007.

147 Chang, H. D. and Radbruch, A., The pro- and antiinflammatory potential of IL-12: the dual role of Th1 cells. *Expert Review of Clinical Immunology* 2007. 3: 709-719.

148 Berghmans, N., Dillen, C. and Heremans, H., Exogenous IL-12 suppresses experimental autoimmune encephalomyelitis (EAE) by tuning IL-10 and IL-5 levels in an IFN-[gamma]-dependent way. *Journal of Neuroimmunology* 2006. 176: 63.

149 Kassner, N., Krueger, M., Yagita, H., Dzionek, A., Hutloff, A., Kroczek, R., Scheffold, A. and Rutz, S., Cutting edge: Plasmacytoid dendritic cells induce IL-10 production in T cells via the Delta-like-4/Notch axis. *J Immunol* 2010. 184: 550-554.

150 Neumann, C., Heinrich, F., Neumann, K., Junghans, V., Mashreghi, M. F., Ahlers, J., Janke, M., Rudolph, C., Mockel-Tenbrinck, N., Kuhl, A. A., Heimesaat, M. M., Esser, C., Im, S. H., Radbruch, A., Rutz, S. and Scheffold, A., Role of Blimp-1 in programing Th effector cells into IL-10 producers. *J Exp Med* 2014. 211: 1807-1819.

151 Pai, S. Y., Truitt, M. L. and Ho, I. C., GATA-3 deficiency abrogates the development and maintenance of T helper type 2 cells. *Proc Natl Acad Sci U S A* 2004. 101: 1993-1998.

152 Hwang, S., Cobb, D. A., Bhadra, R., Youngblood, B. and Khan, I. A., Blimp-1-mediated CD4 T cell exhaustion causes CD8 T cell dysfunction during chronic toxoplasmosis. *J Exp Med* 2016. 213: 1799-1818.

153 Noster, R., de Koning, H. D., Maier, E., Prelog, M., Lainka, E. and Zielinski, C. E., Dysregulation of proinflammatory versus anti-inflammatory human TH17 cell functionalities in the autoinflammatory Schnitzler syndrome. *J Allergy Clin Immunol* 2016.

154 Hebel, K., Rudolph, M., Kosak, B., Chang, H. D., Butzmann, J. and Brunner-Weinzierl, M. C., IL-1beta and TGF-beta act antagonistically in induction and differentially in propagation of human proinflammatory precursor CD4+ T cells. *J Immunol* 2011. 187: 5627-5635.

155 Tokoyoda, K., Zehentmeier, S., Chang, H. D. and Radbruch, A., Organization and maintenance of immunological memory by stroma niches. *Eur J Immunol* 2009. 39: 2095-2099.

156 Neunert, C., Farah, R., Yacobovich, J. and Neufeld, E., Refractory autoimmune disease: an overview of when first-line therapy is not enough. *Semin Hematol* 2016. 53 Suppl 1: S35-38.

157 Schett, G., Emery, P., Tanaka, Y., Burmester, G., Pisetsky, D. S., Naredo, E., Fautrel, B. and van Vollenhoven, R., Tapering biologic and conventional DMARD therapy in rheumatoid arthritis: current evidence and future directions. *Ann Rheum Dis* 2016. 75: 1428-1437.

158 Haschka, J., Englbrecht, M., Hueber, A. J., Manger, B., Kleyer, A., Reiser, M., Finzel, S., Tony, H. P., Kleinert, S., Feuchtenberger, M., Fleck, M., Manger, K., Ochs, W., Schmitt-

Haendle, M., Wendler, J., Schuch, F., Ronneberger, M., Lorenz, H. M., Nuesslein, H., Alten, R., Demary, W., Henes, J., Schett, G. and Rech, J., Relapse rates in patients with rheumatoid arthritis in stable remission tapering or stopping antirheumatic therapy: interim results from the prospective randomised controlled RETRO study. *Ann Rheum Dis* 2016. 75: 45-51.

159 van Amelsfort, J. M., Jacobs, K. M., Bijlsma, J. W., Lafeber, F. P. and Taams, L. S., CD4(+)CD25(+) regulatory T cells in rheumatoid arthritis: differences in the presence, phenotype, and function between peripheral blood and synovial fluid. *Arthritis Rheum* 2004. 50: 2775-2785.

160 Wehrens, E. J., Prakken, B. J. and van Wijk, F., T cells out of control--impaired immune regulation in the inflamed joint. *Nat Rev Rheumatol* 2013. 9: 34-42.

161 de la Rosa, M., Rutz, S., Dorninger, H. and Scheffold, A., Interleukin-2 is essential for CD4+CD25+ regulatory T cell function. *European journal of immunology* 2004. 34: 2480-2488.

162 Brandenburg, S., Takahashi, T., de la Rosa, M., Janke, M., Karsten, G., Muzzulini, T., Orinska, Z., Bulfone-Paus, S. and Scheffold, A., IL-2 induces in vivo suppression by CD4(+)CD25(+)Foxp3(+) regulatory T cells. *European journal of immunology* 2008. 38: 1643-1653.

163 Hwang, E. S., Hong, J. H. and Glimcher, L. H., IL-2 production in developing Th1 cells is regulated by heterodimerization of RelA and T-bet and requires T-bet serine residue 508. *J Exp Med* 2005. 202: 1289-1300.

164 Humrich, J. Y., Morbach, H., Undeutsch, R., Enghard, P., Rosenberger, S., Weigert, O., Kloke, L., Heimann, J., Gaber, T., Brandenburg, S., Scheffold, A., Huehn, J., Radbruch, A., Burmester, G. R. and Riemekasten, G., Homeostatic imbalance of regulatory and effector T cells due to IL-2 deprivation amplifies murine lupus. *Proc Natl Acad Sci U S A* 2010. 107: 204-209.

165 von Spee-Mayer, C., Siegert, E., Abdirama, D., Rose, A., Klaus, A., Alexander, T., Enghard, P., Sawitzki, B., Hiepe, F., Radbruch, A., Burmester, G. R., Riemekasten, G. and Humrich, J. Y., Low-dose interleukin-2 selectively corrects regulatory T cell defects in patients with systemic lupus erythematosus. *Ann Rheum Dis* 2015.

166 Humrich, J. Y., von Spee-Mayer, C., Siegert, E., Alexander, T., Hiepe, F., Radbruch, A., Burmester, G. R. and Riemekasten, G., Rapid induction of clinical remission by low-dose interleukin-2 in a patient with refractory SLE. *Ann Rheum Dis* 2015. 74: 791-792.

167 Niesner, U., Albrecht, I., Janke, M., Doebis, C., Loddenkemper, C., Lexberg, M. H., Eulenburg, K., Kreher, S., Koeck, J., Baumgrass, R., Bonhagen, K., Kamradt, T., Enghard, P., Humrich, J. Y., Rutz, S., Schulze-Topphoff, U., Aktas, O., Bartfeld, S., Radbruch, H., Hegazy, A. N., Lohning, M., Baumgart, D. C., Duchmann, R., Rudwaleit, M., Haupl, T., Gitelman, I., Krenn, V., Gruen, J., Sieper, J., Zeitz, M., Wiedenmann, B., Zipp, F., Hamann,

A., Janitz, M., Scheffold, A., Burmester, G. R., Chang, H. D. and Radbruch, A., Autoregulation of Th1-mediated inflammation by twist1. *J Exp Med* 2008. 205: 1889-1901.

168 Albrecht, I., Niesner, U., Janke, M., Menning, A., Loddenkemper, C., Kuhl, A. A., Lepenies, I., Lexberg, M. H., Westendorf, K., Hradilkova, K., Grun, J., Hamann, A., Epstein, J. A., Chang, H. D., Tokoyoda, K. and Radbruch, A., Persistence of effector memory Th1 cells is regulated by Hopx. *Eur J Immunol* 2010. 40: 2993-3006.

169 Sosic, D., Richardson, J. A., Yu, K., Ornitz, D. M. and Olson, E. N., Twist regulates cytokine gene expression through a negative feedback loop that represses NF-kappaB activity. *Cell* 2003. 112: 169-180.

170 Sharif, M. N., Sosic, D., Rothlin, C. V., Kelly, E., Lemke, G., Olson, E. N. and Ivashkiv, L. B., Twist mediates suppression of inflammation by type I IFNs and Axl. *The Journal of experimental medicine* 2006. 203: 1891-1901.

171 Pham, D., Vincentz, J. W., Firulli, A. B. and Kaplan, M. H., Twist1 regulates Ifng expression in Th1 cells by interfering with Runx3 function. *J Immunol* 2012. 189: 832-840.

172 Wojciechowski, S., Tripathi, P., Bourdeau, T., Acero, L., Grimes, H. L., Katz, J. D., Finkelman, F. D. and Hildeman, D. A., Bim/Bcl-2 balance is critical for maintaining naive and memory T cell homeostasis. *J Exp Med* 2007. 204: 1665-1675.

173 Shin, C. H., Liu, Z. P., Passier, R., Zhang, C. L., Wang, D. Z., Harris, T. M., Yamagishi, H., Richardson, J. A., Childs, G. and Olson, E. N., Modulation of cardiac growth and development by HOP, an unusual homeodomain protein. *Cell* 2002. 110: 725-735.

174 Chen, F., Kook, H., Milewski, R., Gitler, A. D., Lu, M. M., Li, J., Nazarian, R., Schnepp, R., Jen, K., Biben, C., Runke, G., Mackay, J. P., Novotny, J., Schwartz, R. J., Harvey, R. P., Mullins, M. C. and Epstein, J. A., Hop is an unusual homeobox gene that modulates cardiac development. *Cell* 2002. 110: 713-723.

175 Goldblatt, F. and Isenberg, D. A., New therapies for rheumatoid arthritis. *Clinical and experimental immunology* 2005. 140: 195-204.

176 Farge, D., Labopin, M., Tyndall, A., Fassas, A., Mancardi, G. L., Van Laar, J., Ouyang, J., Kozak, T., Moore, J., Kotter, I., Chesnel, V., Marmont, A., Gratwohl, A. and Saccardi, R., Autologous hematopoietic stem cell transplantation (HSCT) for autoimmune diseases: an observational study on 12 years of experience from the European Group for Blood and Marrow Transplantation (EBMT) Working Party on Autoimmune Diseases. *Haematologica* 2009.

177 Alexander, T., Thiel, A., Rosen, O., Massenkeil, G., Sattler, A., Kohler, S., Mei, H., Radtke, H., Gromnica-Ihle, E., Burmester, G. R., Arnold, R., Radbruch, A. and Hiepe, F., Depletion of autoreactive immunologic memory followed by autologous hematopoietic stem cell transplantation in patients with refractory SLE induces long-term remission through de novo generation of a juvenile and tolerant immune system. *Blood* 2009. 113: 214-223.

178 Muraro, P. A., Douek, D. C., Packer, A., Chung, K., Guenaga, F. J., Cassiani-Ingoni, R., Campbell, C., Memon, S., Nagle, J. W., Hakim, F. T., Gress, R. E., McFarland, H. F., Burt, R. K. and Martin, R., Thymic output generates a new and diverse TCR repertoire after autologous stem cell transplantation in multiple sclerosis patients. *J Exp Med* 2005. 201: 805-816.

179 Vallerskog, T., Gunnarsson, I., Widhe, M., Risselada, A., Klareskog, L., van Vollenhoven, R., Malmstrom, V. and Trollmo, C., Treatment with rituximab affects both the cellular and the humoral arm of the immune system in patients with SLE. *Clin Immunol* 2007. 122: 62-74.

180 Cambridge, G., Leandro, M. J., Teodorescu, M., Manson, J., Rahman, A., Isenberg, D. A. and Edwards, J. C., B cell depletion therapy in systemic lupus erythematosus: effect on autoantibody and antimicrobial antibody profiles. *Arthritis Rheum* 2006. 54: 3612-3622.

181 Alexander, T., Arnold, R., Hiepe, F. and Radbruch, A., Resetting the immune system with immunoablation and autologous haematopoietic stem cell transplantation in autoimmune diseases. *Clin Exp Rheumatol* 2016. 34: 53-57.

182 Hoyer, B. F., Mumtaz, I. M., Yoshida, T., Hiepe, F. and Radbruch, A., How to cope with pathogenic long-lived plasma cells in autoimmune diseases. *Annals of the rheumatic diseases* 2008. 67 Suppl 3: iii87-89.

183 Neubert, K., Meister, S., Moser, K., Weisel, F., Maseda, D., Amann, K., Wiethe, C., Winkler, T. H., Kalden, J. R., Manz, R. A. and Voll, R. E., The proteasome inhibitor bortezomib depletes plasma cells and protects mice with lupus-like disease from nephritis. *Nat Med* 2008. 14: 748-755.

184 Alexander, T., Sarfert, R., Klotsche, J., Kuhl, A. A., Rubbert-Roth, A., Lorenz, H. M., Rech, J., Hoyer, B. F., Cheng, Q., Waka, A., Taddeo, A., Wiesener, M., Schett, G., Burmester, G. R., Radbruch, A., Hiepe, F. and Voll, R. E., The proteasome inhibitior bortezomib depletes plasma cells and ameliorates clinical manifestations of refractory systemic lupus erythematosus. *Ann Rheum Dis* 2015. 74: 1474-1478.

185 Taddeo, A., Gerl, V., Hoyer, B. F., Chang, H. D., Kohler, S., Schaffert, H., Thiel, A., Radbruch, A. and Hiepe, F., Selection and depletion of plasma cells based on the specificity of the secreted antibody. *Eur J Immunol* 2015. 45: 317-319.

186 Herzog, C., Walker, C., Muller, W., Rieber, P., Reiter, C., Riethmuller, G., Wassmer, P., Stockinger, H., Madic, O. and Pichler, W. J., Anti-CD4 antibody treatment of patients with rheumatoid arthritis: I. Effect on clinical course and circulating T cells. *Journal of autoimmunity* 1989. 2: 627-642.

187 Tak, P. P., van der Lubbe, P. A., Cauli, A., Daha, M. R., Smeets, T. J., Kluin, P. M., Meinders, A. E., Yanni, G., Panayi, G. S. and Breedveld, F. C., Reduction of synovial inflammation after anti-CD4 monoclonal antibody treatment in early rheumatoid arthritis. *Arthritis Rheum* 1995. 38: 1457-1465.

188 Canva-Delcambre, V., Jacquot, S., Robinet, E., Lemann, M., Drouet, C., Labalette, M., Dessaint, J. P., Bengoufa, D., Rabian, C., Modigliani, R., Wijdenes, J., Revillard, J. P. and Colombel, J. F., Treatment of severe Crohn's disease with anti-CD4 monoclonal antibody. *Aliment Pharmacol Ther* 1996. 10: 721-727.

189 Lenschow, D. J., Zeng, Y., Thistlethwaite, J. R., Montag, A., Brady, W., Gibson, M. G., Linsley, P. S. and Bluestone, J. A., Long-term survival of xenogeneic pancreatic islet grafts induced by CTLA4lg. *Science* 1992. 257: 789-792.

190 Kremer, J. M., Westhovens, R., Leon, M., Di Giorgio, E., Alten, R., Steinfeld, S., Russell, A., Dougados, M., Emery, P., Nuamah, I. F., Williams, G. R., Becker, J. C., Hagerty, D. T. and Moreland, L. W., Treatment of rheumatoid arthritis by selective inhibition of T-cell activation with fusion protein CTLA4lg. *N Engl J Med* 2003. 349: 1907-1915.

191 Genovese, M. C., Becker, J. C., Schiff, M., Luggen, M., Sherrer, Y., Kremer, J., Birbara, C., Box, J., Natarajan, K., Nuamah, I., Li, T., Aranda, R., Hagerty, D. T. and Dougados, M., Abatacept for rheumatoid arthritis refractory to tumor necrosis factor alpha inhibition. *N Engl J Med* 2005. 353: 1114-1123.

192 Crnkic Kapetanovic, M., Saxne, T., Jonsson, G., Truedsson, L. and Geborek, P., Rituximab and abatacept but not tocilizumab impair antibody response to pneumococcal conjugate vaccine in patients with rheumatoid arthritis. *Arthritis Res Ther* 2013. 15: R171.

193 Stittrich, A. B., Haftmann, C., Sgouroudis, E., Kuhl, A. A., Hegazy, A. N., Panse, I., Riedel, R., Flossdorf, M., Dong, J., Fuhrmann, F., Heinz, G. A., Fang, Z., Li, N., Bissels, U., Hatam, F., Jahn, A., Hammoud, B., Matz, M., Schulze, F. M., Baumgrass, R., Bosio, A., Mollenkopf, H. J., Grun, J., Thiel, A., Chen, W., Hofer, T., Loddenkemper, C., Lohning, M., Chang, H. D., Rajewsky, N., Radbruch, A. and Mashreghi, M. F., The microRNA miR-182 is induced by IL-2 and promotes clonal expansion of activated helper T lymphocytes. *Nat Immunol* 2010. 11: 1057-1062.

194 Haftmann, C., Riedel, R., Porstner, M., Wittmann, J., Chang, H. D., Radbruch, A. and Mashreghi, M. F., Direct uptake of Antagomirs and efficient knockdown of miRNA in primary B and T lymphocytes. *J Immunol Methods* 2015. 426: 128-133.